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14. ABSTRACT Genomic instability is a hallmark of breast cancer cells. DNA damage checkpoints are critical for the prevention of genomic instability and breast cancer. The ATR checkpoint kinase is activated in response to exogenous and endogenous DNA damage, and phosphorylates downsteam substrates such as BRCA1 and p53 to promote cell cycle arrest DNA repair, and apoptosis. ATR exists in a complex with ATR-interacting protein (ATRIP). In response to DNA damage, the ATR-ATRIP complex is recruited to DNA lesions in part through an interaction between ATRIP and the single-stranded DNA binding protein RPA (Replication Protein A). We report the identification of a conserved checkpoint protein recruitment domain (CRD) in ATRIP orthologs by biochemical mapping of the RPA binding site in combination with NMR, mutagenesis, and computational modeling studies. Mutations in the CRD of the yeast ATRIP ortholog Ddc2 disrupt the Ddc2-RPA interaction, prevent proper localization of Ddc2 to DNA breaks, sensitize yeast to DNA damaging agents, and partially compromise checkpoint signaling. We have also defined a conserved TopBP1 interacting region in ATRIP that is necessary for ATR activation. Finally, we have discovered a PIKK regulatory domain (PRD) in ATR that is required for its activation by TopBP1 and cellular viability. Thus, our results support a multi-step model for ATR activation that requires separable localization and activation functions of ATRIP, and helps to explain how cells maintain genome stability and prevent tumorigenesis.

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Introduction:

The human genome is constantly subject to DNA damaging agents, including mutagens from the environment, such as ultraviolet radiation, and by-products of cellular metabolism, such as free oxygen radicals. The DNA damage response is a multifaceted signaling pathway that protects the genome and forms an anti-cancer barrier, including eliminating cells with extensive DNA damage and preventing the proliferation of precancerous lesions (1). Defects in many of the proteins in this pathway, including ATM and BRCA1, are associated with breast cancer predisposition syndromes (2). The DNA damage activated kinase ATR is the key regulator of the DNA damage response, and phosphorylates many tumor suppressor proteins, such as p53 and BRCA1 (3). Although many of the downstream targets of ATR have been studies, the mechanism of ATR activation has remained elusive (2). ATR exists as a stable complex with an associated protein ATRIP (ATR Interacting Protein), which promotes its recruitment to sites of DNA damage and is required for ATR signaling (4-6). To understand how ATR is regulated, we have studied the functional roles of ATRIP and its yeast ortholog Ddc2. We have examined the interactions between ATRIP and two other proteins essential for the DNA damage response, RPA (Replication Protein A) and TopBP1 (Topoisomerase II-β binding protein 1) (7, 8). Elucidation of the mechanisms of activation of ATR is critical to understanding how cells prevent breast cancer, how genotoxic breast cancer therapies work, and how better therapeutic agents that target this pathway can be developed.

Body:

Regulation of ATR-ATRIP localization by the single-stranded DNA binding protein RPA
(also see Appendix A)

ATR-ATRIP is located diffusely throughout the nucleus in undamaged cells. Following exposure of cells to DNA damage, ATR-ATRIP relocalizes to accumulate into distinct nuclear foci that co-localization with other checkpoint and repair proteins as well as phosphorylated γ-H2AX, a marker for DNA damage (9, 10). ATR-ATRIP relocalization to foci requires an interaction between ATRIP and RPA-ssDNA (11). DNA damage perturbs DNA replication, leading to the generation of abnormally large amounts of RPA-coated single-stranded DNA (12). For this reason, RPA-ssDNA is thought to be a trigger for ATR activation (12, 13). We identified an N-terminal ATRIP domain required for binding RPA-ssDNA. Deletion of this N-terminal RPA binding domain prevented the accumulation of ATR-ATRIP into nuclear foci, but, surprisingly had no effect on ATR-dependent phosphorylation of its downstream substrate Chk1 (11). These results suggested that stable retention of ATR at a damage site is not required for ATR activation in response to UV, HU and IR (11).

We have furthered our understanding of this process through the completion of this grant by examining the role of ATRIP localization to sites of DNA damage in ATR activation. Using a combination of biochemical mapping, NMR, mutagenesis and computational modeling we have identified a conserved checkpoint protein recruitment domain (CRD) in ATRIP orthologs. Mutations in the CRD of the yeast ATRIP ortholog Ddc2 disrupt the Ddc2-RPA interaction, prevent proper localization of Ddc2 to DNA breaks, sensitize yeast to DNA damaging agents, and partially compromise checkpoint signaling (14). These data demonstrate that the CRD is critical for localization and optimal DNA damage responses. Recently, Kumagai et al. discovered that a fragment of TopBP1 could activate ATR both in vitro and in vivo. This TopBP1-dependent activation of ATR

is ATRIP-dependent, but does not require the ATRIP CRD (14, 15), suggesting that ATRIP contains separate domains involved in RPA-binding dependent recruitment of ATR-ATRIP to DNA lesions and TopBP1-dependent activation of the ATR kinase. Our results, in combination with others, support a multi-step model for ATR activation that requires separable localization and activation functions of ATRIP

Regulation of ATR-ATRIP activation by the ATR activator protein TopPB1

(also see appendix B)

TopBP1 is an eight BRCT repeat protein with essential roles in DNA replication and checkpoint signaling. TopBP1 stimulates the kinase activity of the ATR-ATRIP complex but not ATM (15). However, the mechanism(s) by which TopBP1 promotes ATR signaling is not well understood. We have defined how TopBP1 interacts with the ATR-ATRIP complex. Recently, we have also examined the regulation of Mec1-Ddc2, the *S. cerevisiae* orthologs of ATR-ATRIP.

The region responsible for this function of TopBP1 has been mapped to a 308 amino acid domain between BRCT repeat 6 and 7 called the ATR Activation Domain, AAD (15). We and others have demonstrated that the TopBP1 AAD is sufficient to activate ATR *in vitro* and *in vivo* (see included manuscript) (15, 16). The only reported physical interaction between these proteins comes from work performed using the *in vitro* xenopus egg extract system that demonstrated an interaction between the TopBP1 AAD and the ATR-ATRIP complex (15). To begin to map the interaction surfaces between TopBP1 and the ATRIP-ATR complex, we took a yeast-two hybrid approach. The TopBP1 AAD fused to the GAL4 DNA binding domain as bait was used to screen libraries of random fragments of ATR and ATRIP between 100 and 500 amino acids in length fused to the activation

domain of GAL4. These libraries have been previously used to map the ATR-ATRIP interaction domains (5). Our analysis of ATRIP fragments capable of an interaction with TopBP1 revealed that all fragments contained at least amino acids 204-398. Aside from containing a small part of the coiled-coil domain of ATRIP, the function of this region is unknown. Preliminary data from our lab has found that this region contains multiple phosphorylation sites, yet the significance of these phosphorylation sites remains to be determined.

We also had positive hits in the screen of the ATR fragment library. All clones mapped to the extreme C-terminus of ATR with the minimal domain of interaction being amino acids 2483-2597. This region of ATR contains approximately 60 amino acids of the C-terminal end of the kinase domain and a highly conserved adjacent region of unknown function. Since TopBP1 greatly stimulates the kinase activity of ATR it seemed plausible that TopBP1 would bind to the kinase domain or an adjacent region. We proposed that the region between the kinase domain and FATC domain is a regulatory domain of ATR, called the PRD (PIKK regulatory domain) (see below) that mediates its interaction with TopBP1. In support of this idea, a charge-reversal point mutation in this domain severely abrogates TopBP1 stimulation of ATR kinase activity towards a substrate *in vitro* (Figure 1).

Using recombinant fragments of TopBP1, we were able to demonstrate that endogenous ATR-ATRIP from nuclear extracts specifically binds to fragments containing the AAD but not the C-terminal BRCT repeats of TopBP1 (Figure 2). As expected, the related kinase ATM does not bind to any fragments of TopBP1. This assay was used to define the region of interaction between TopBP1 and ATR-ATRIP.

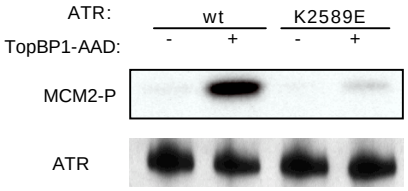

Figure 1: The ATR-ATRIP complex was purified from cells expressing either wild-type (wt) or mutant ATR and incubated with radioactive ATP and a fragment of MCM2, an ATR substrate, with or without the TopBP1 AAD. The phosphorylation of the substrate was detected using autoradiography. A western blot against ATR indicates equal kinase amounts.

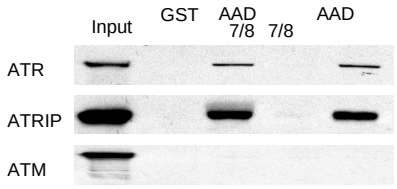

Figure 2: Nuclear extracts from mammalian cells were incubated with beads containing recombinant GST-tagged TopBP1 fragments. The beads were washed and the bound proteins were detected using Western blotting with the indicated antibodies. GST- control, 7/8- BRCT repeat 7&8.

Characterization of the interaction between TopBP1 and ATRIP

Defining how TopBP1 interacts with ATR-ATRIP is critical to understanding to the mechanism of ATR activation. We have demonstrated that ATRIP promotes the association of ATR and TopBP1. We have defined a TopBP1 interacting region of ATRIP. In vitro studies revealed that mutation of this region decreased the ability of TopBP1 to associate with the ATR-ATRIP complex and inhibited the ability of TopBP1 to stimulate ATR kinase activity. Cell based studies indicated that the interaction between ATRIP and TopBP1 was necessary for cellular checkpoint responses, including recovery of DNA replication after replication stress and the G2/M cell cycle checkpoint. We conclude that the interaction between ATRIP and TopBP1 is required for ATR to appropriately respond to DNA damage and replication stress. Finally, we have demonstrated that this region of ATRIP is conserved in the *S. cerevisiae* ortholog Ddc2 as described below.

Investigation of the function of the ATR PIK Kinase Regulatory Domain (PRD)

We have also examined the interaction between TopBP1 and ATR. We have defined a TopBP1 interacting region of ATR that mapped to an uncharacterized C-terminal region between the kinase domain and FATC domain. We have named this region the PIK Kinase Regulatory Domain (PRD) since the analogous region in other PIK kinases has been shown

to be important for their regulation. Mutations of this domain did not affect the basal kinase activity of ATR. Specific mutations in the ATR PRD abrogated TopBP1-dependent stimulation. Cell based complementation experiments using $ATR^{flox/-}$ cells revealed that disruption of the PRD resulted in defective ATR-dependent checkpoint signaling.

Furthermore, we could not isolate cells expressing only the PRD mutant form of ATR. This suggests that TopBP1-dependent activation of ATR is required for cellular viability even in the absence of exogenous DNA damage.

We have performed additional analysis of the PRD. Recently, an acetylation site of ATM has been mapped to the ATM KRD. Acetylation of ATM by the Tip60 histone acetylase complex was shown to be essential for ATM activation after DNA damage but not its basal kinase activity. We hypothesized that ATR acetylation may be necessary for its stimulation by TopBP1. Mutations of all ATR PRD lysine residues to arginines did not affect the TopBP1 stimulation of ATR, and siRNA depletion of Tip60 did not cause sensitivity to replication stress. Thus, we conclude that ATR is not regulated by acetylation in the same manner as ATM.

The PRD has been shown to be important for the regulation of ATM and mTOR (17, 18). We examined the PRD of another PIKK family member DNA-PKcs (DNA-activated protein kinase catalytic subunit). Select mutations in the PRD of DNA-PKcs resulted in the loss of DNA-PKcs autophosphorylation, a marker of DNA-PKcs activation. This suggests that the PRD is necessary for the regulation of most, if not all, PIKK family members, and that the mechanism of activation may be similar among PIKKs.

Analysis of the regulation of the *S. cerevisiae* Mec1-Ddc2 (ATR-ATRIP) complex
(also see appendix C)

Most checkpoint proteins and activities are conserved in all eukaryotic cells. In *Saccharomyces cerevisiae,* the orthologs of ATR and ATRIP are Mec1 and Ddc2 (3).

Although TopBP1 has been shown to activate ATR, it is not clear how Mec1 becomes activated. Both Ddc1 (Rad9 homolog) and Dpb11 (TopBP1 homolog) have been proposed to function as the Mec1 activator protein (7, 19). We have found that a mutation in Ddc2 (termed ddc2-top) in a location analogous to the TopBP1 interacting region of ATRIP resulted in sensitivity to replication stress and DNA damage. Moreover, ddc2-top yeast exhibited defects in checkpoint signaling after replication stress and DNA damage. We hypothesized that the ddc2-top mutation compromised an interaction between Ddc2 and the Mec1 activator. We found that overexpression of Dpb11 but not Ddc1 partially suppressed the sensitivity of ddc2-top to replication stress, suggesting an interaction between Dpb11 and Ddc2.

We have defined a domain of Dpb11 that interacts with the Mec1-Ddc2 complex but not Mec1-ddc2-top. More importantly, we have shown that this domain of Dpb11 stimulates the kinase activity of wild-type Mec1-Ddc2 yet did not simulate Mec1-ddc2-top. Thus, we have demonstrated that Dpb11 function as a Mec1 activator and that the mechanism of ATR activation is likely conserved throughout evolution.

Key Research Accomplishments:

-Defined how the ATRIP interacts with RPA-single stranded DNA

-Created a structural model of the interaction between the ATRIP checkpoint recruitment domain (CRD) and the 70N domain of RPA

-Demonstrated that the ATRIP CRD was conserved in the ATRIP budding yeast ortholog Ddc2

-Characterized the function of the ATRIP CRD in yeast in terms of localization, resistance to DNA damaging agents, and checkpoint signaling

-Established kinase assay protocols to measure the activation of ATR and the ATR budding yeast ortholog Mec1

-Demonstrated that the stimulation of ATR kinase activity by TopBP1 can occur independently of the interaction between ATRIP and RPA

-Identified a TopBP1 interacting region in ATRIP and showed that this region is required for ATR activation and cellular checkpoint response to DNA damage

-Revealed that the TopBP1 interacting region is functionally conserved in the ATRIP ortholog Ddc2

-Discovered that the TopBP1 budding yeast homolog Dpb11 could activate the Mec1-Ddc2 complex (ATR-ATRIP)

-Established that the FATC domain of ATR and all other DNA damage activated kinase is required for its kinase activity

-Discovered a separation-of-function mutant in ATR that specifically impairs it activation by TopBP1 and the ATR signaling pathway

-Use similarities among different DNA damage activated kinases to uncover a PIKK regulatory domain (PRD) that is important for the function of ATR, ATM, and DNA-PKcs

-Showed that the PRD of DNA-PKcs was important for its response to DNA damage

Reportable Outcomes:

Heather L. Ball

-Presentation of work at International Ataxia Telangiectasia Workshop, September 2006, entitled "Role of a Conserved RPA-ATRIP Interaction in ATR Signaling".

-Publication of manuscript "Function of a conserved checkpoint recruitment domain in ATRIP proteins" in Molecular & Cellular Biology, March 2007.

- Defense of PhD thesis on March 30th, 2007 for this and previous work.

- Post-doctoral research position secured at Hospital for Sick Kids, University of Toronto, Toronto, Ontario.

Daniel A. Mordes

-Presentation of work at Cold Spring Harbor Laboratory Meeting: Eukaryotic DNA Replication & Genome Maintenance, September 2007, entitled "Regulation of the Checkpoint Kinase ATR by TopBP1".

-Presentation of work at International Ataxia Telangiectasia Workshop, April 2008, entitled "TopBP1 Activates ATR through ATRIP and a PIKK Regulatory Domain".

-Publication of manuscript "TopBP1 Activates ATR through ATRIP and a PIKK Regulatory Domain" in Genes and Development, June 2008.

-Submitted manuscript "Dpb11 activates the Mec1-Ddc2 complex" to PNAS, July 2008.

-Scheduled PhD thesis defense for September 12th, 2008 for this and previous work.

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3. Mordes DA & Cortez D (unpublished) Dpb11 activates the Mec1-Ddc2 complex. *submitted July 2008*.

Conclusions:

The ATR-ATRIP kinase complex is activated by DNA damage and coordinates numerous cellular responses to preserve genomic stability. We have examined the regulation of ATR-ATRIP complex at multiple levels. We have demonstrated that ATR activation requires its localization to sites of DNA damage, in part due to a direct interaction between ATRIP and RPA, and its binding to the TopBP1 activator protein. ATRIP was found to contain a conserved checkpoint recruitment domain that was necessary for the proper localization to DNA damage and the ATR-dependent DNA damage response. Besides ensuring the proper localization of ATR, we found that ATRIP also promotes the association between TopBP1 and ATR. We identified a conserved TopBP1 interacting region of ATRIP that was necessary for ATR activation and important for the DNA damage response. Moreover, we have demonstrated that the mechanism of ATR activation is conserved from yeast to humans. Finally, we discovered a PIKK regulatory domain that is important for the activation of ATR as well as other related DNA damage activated kinases, including DNA-PKcs and ATM.

Genomic instability is a hallmark of breast cancer cells. The ATR/ATM-mediated DNA damage response promotes preservation of the genome and has been postulated to act as an anti-cancer barrier that prevents the proliferation of precancerous cells. For example, activation of the ATM is evident in ductal carcinoma *in situ*, a precursor lesion to breast cancer (20). ATM is responsible for inducing cellular senescence in response to oncogene activation (21). Both ATM and ATR are key regulators of many breast cancer tumor suppressor proteins, including BRCA1, p53, and FANCD2. Disruptions in the ATR/ATM pathways are linked to breast cancer. Heterozygous carriers for mutations in *ATM*, which includes approximately 1% of North American women, have about a two-fold increased

risk of breast cancer (22). Inherited mutations in *Chk2*, a key substrate of ATM, also are associated with an increased risk of breast cancer (23).

TopBP1 also likely has functions in preserving the genome similar to those of BRCA1. The C-terminal third of TopBP1 shares 35% sequence similarity and 25% sequence identity to the C-terminal of BRCA1, which is important for its tumor suppressor function (24, 25). Both proteins are phosphorylated at multiple sites by ATM after DNA damage (26, 27). In fact, IR induces the formation of a protein complex containing TopBP1 and BRCA1 that is involved in checkpoint signaling (28). A study using breast carcinoma cells showed that cells deficient in TopBP1 or deficient in BRCA1 showed reduced activation of the ATR checkpoint signaling pathway (29). A polymorphism in *TopBP1* is associated with an increased risk of hereditary breast and/or ovarian cancer, and TopBP1 is mislocalized to the cytoplasm in some breast carcinomas (30, 31). Although *TopBP1* has been proposed to be a breast cancer susceptibility gene, further studies are necessary to determine the link between TopBP1 and breast cancer.

Many breast cancer treatments including chemotherapeutic drugs, such as anthracyclines and cyclophosphamide, and ionizing radiation act to cause DNA damage and activate the ATR/ATM pathways. Cancerous cells are thought to have defects in DNA repair and DNA damage response pathways that make them more susceptible than normal cells to these treatments. Thus, additional treatments that target the DNA damage pathway are being develop as chemo- or radio-sensitizing agents. For example, inhibition of ATR function sensitizes several cancer cell lines to both alkylating and radiomimetic agents(32). Overall, elucidation of the mechanisms of activation of ATR and ATM checkpoint pathways is critical to understanding both the etiology of breast cancer and mode of action of many common breast cancer therapeutic modalities.

Furthermore, the molecular characterization of ATR and ATM activation may be useful in the development of pharmacological agents that target these kinases. Inhibition of ATR sensitizes cancer cells to multiple DNA damaging agents (32). Thus far, specific inhibitors of ATM and DNA-PKcs kinases have been developed and are being studied as potential therapeutic agents (33); however, inhibitors of the ATR kinase have not been isolated. Targeting the interaction between ATRIP and RPA, ATRIP and TopBP1, or between the ATR PRD and TopBP1 may provide a novel means for developing an agent to disrupt ATR signaling.

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Appendices:

A. Manuscript “Function of a conserved checkpoint recruitment domain in ATRIP proteins”
B. Manuscript “TopBP1 activates ATR through ATRIP and a PIKK regulatory domain”
C. Manuscript “Dpb11 activates the Mec1-Ddc2 complex” (unpublished data)

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Function of a Conserved Checkpoint Recruitment Domain in ATRIP Proteins▽

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The ATR (ATM and Rad3-related) kinase is essential to maintain genomic integrity. ATR is recruited to DNA lesions in part through its association with ATR-interacting protein (ATRIP), which in turn interacts with the single-stranded DNA binding protein RPA (replication protein A). In this study, a conserved *c*heckpoint protein *r*ecruitment *d*omain (CRD) in ATRIP orthologs was identified by biochemical mapping of the RPA binding site in combination with nuclear magnetic resonance, mutagenesis, and computational modeling. Mutations in the CRD of the *Saccharomyces cerevisiae* ATRIP ortholog Ddc2 disrupt the Ddc2-RPA interaction, prevent proper localization of Ddc2 to DNA breaks, sensitize yeast to DNA-damaging agents, and partially compromise checkpoint signaling. These data demonstrate that the CRD is critical for localization and optimal DNA damage responses. However, the stimulation of ATR kinase activity by binding of topoisomerase binding protein 1 (TopBP1) to ATRIP-ATR can occur independently of the interaction of ATRIP with RPA. Our results support the idea of a multistep model for ATR activation that requires separable localization and activation functions of ATRIP.

ATR (ATM and Rad3-related) kinase is a protein kinase that coordinates cellular responses to genotoxic stress. ATR activation occurs primarily in S phase due to replication stress induced by DNA-damaging agents or replication inhibitors. More specifically, ATR activation is stimulated when the replication machinery encounters a DNA lesion and becomes uncoupled (the helicase continues to unwind DNA while the polymerase becomes stalled at the site of DNA damage) (9).

The critical factor that promotes ATR activation is believed to be the accumulation of RPA (replication protein A)-coated single-stranded DNA (ssDNA) (11, 33, 43). At least two separate checkpoint complexes accumulate in distinct foci that colocalize with RPA. Rad17, a PCNA-like clamp loader protein, is recruited to RPA-ssDNA and loads the Rad9-Rad1-Hus1 checkpoint clamp at the junction of double-stranded and single-stranded DNA (4, 14, 53). Independently, ATR is recruited by ATR-interacting protein (ATRIP), which binds the RPA-ssDNA that accumulates at DNA lesions (3, 15, 37, 52).

ATRIP is required for ATR function, and mutation of either ATR or ATRIP causes the same phenotypes (3, 12). The strict requirement for ATRIP is conserved in *Schizosaccharomyces pombe* (Rad3 and Rad26), *Saccharomyces cerevisiae* (Mec1 and Ddc2/Lcd1/Pie1), and *Xenopus laevis* (xATR and xATRIP) (13, 38, 41, 51). An N-terminal domain of ATRIP binds RPA-ssDNA and is necessary for stable ATR-ATRIP localization to damage-induced nuclear foci (3, 25).

The ATR signaling pathway is currently viewed as an important target for the development of cancer therapies (10, 22, 24, 32, 34). However, the mechanism by which ATR is activated remains unclear. Localization to sites of DNA damage or replication stress has been suggested to be essential and perhaps sufficient to promote ATR signaling. However, mutations in ATRIP that disrupt the stable RPA-ATRIP interaction and impair the accumulation of ATR-ATRIP complexes in DNA-damage-induced foci have minimal effects on ATR activation and signaling (3, 25). Furthermore, topoisomerase binding protein 1 (TopBP1) was recently discovered to stimulate ATR kinase activity, suggesting regulation by a means other than localization (28). To clarify the functions of ATRIP, RPA, and TopBP1 in mediating ATR-dependent checkpoint response we have performed a series of biochemical and genetic experiments in human and yeast systems. We report structural and functional data that support a model for ATR activation in which two separable ATRIP activities—localization and activation—cooperate to promote ATR signaling.

MATERIALS AND METHODS

Yeast strains. All strains used in this study are described in Table 1. *Myc-Ddc2* and *Myc-Ddc2* mutant strains were generated by expressing mutants from a centromeric plasmid under the control of the endogenous *DDC2* promoter in strain DMP2995/1B (*MAT***a** *sml1D*::*KanMX4 ddc2D*::*KanMX4*) (38). *GFP-Ddc2ΔN* was generated in JK8-1 (36) by use of the delitto perfetto system (47). Strain yHB244 was generated by expressing RNR3 by use of pBAD79 and deleting *DDC2* by use of pGEM499 in the JKM179 strain (30). *Myc-DDC2* and *Myc-Ddc2ΔN* were expressed in strain yHB244 from the pNML1 centromeric plasmid (42).

RPA-ssDNA and RPA binding. The 14 kDa and 70 kDa RPA subunits were tagged with an His_6 epitope tag (45). RPA was purified from *Escherichia coli* by use of nickel affinity chromatography followed by Superdex fractionation. A 20-pmol volume of biotin-labeled 69-bp single-stranded oligonucleotide was bound to streptavidin beads and incubated with binding buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 10% glycerol, 0.02% Igepal CA-630, 10 μg/ml bovine serum albumin) alone or with a 4 M excess of RPA in binding buffer. The RPA-ssDNA-streptavidin beads were washed three times with binding buffer prior to use. Hemagglutinin-ATRIP (HA-ATRIP) fragments were generated using in vitro

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TABLE 1. Yeast strains used in this study

Strain	Description-genotype	Reference or source
DMP2995/1B	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sml1*::*KanMX4*	38
yHB200	DMP2995/1B [p1220: *Myc-URA3-CEN*]	This study
yHB201	DMP2995/1B [pNML1: *Myc-DDC2-URA3-CEN*]	This study
yHB202	DMP2995/1B [pHB126: *Myc-ddc2ΔN-URA3-CEN*]	This study
yHB221	DMP2995/1B [pHB157: *Myc-ddc2D12KD13K-URA3-CEN*]	This study
yHB222	DMP2995/1B [pHB155: *Myc-ddc2NAAIRS14-19-URA3-CEN*]	This study
Rfa1-TAP	*MAT***a** *his3-1 leu2-0 met15-0 ura3-0 Rfa1-TAP*	17
yHB231	Rfa1-TAP [pNML1: *Myc-DDC2-URA-CEN*]	This study
yHB232	Rfa1-TAP [pHB126: *Myc-ddc2ΔN-URA-CEN*]	This study
yHB233	Rfa1-TAP [pHB157: *Myc-ddc2D12KD13K-URA3-CEN*]	This study
yHB234	Rfa1-TAP [pHB155: *Myc-ddc2NAAIRS14-19-URA3-CEN*]	This study
JKM179	*hoΔ MAT***a** *hmlΔ*::*ADE1 hmrΔ*::*ADE1 ade1-100 leu2-3,112 lys5 trp1*::*hisG′ ura3-52 ade3*::*GAL*::*HO*	29
yHB244	JKM179 *ddc2Δ*::*LEU2* [pBAD79: *RNR3-TRP1-CEN*]	This study
yHB245	yHB244 (GAL-HO, Δ*ddc2*) [p1220: *Myc-URA3-CEN*]	This study
yHB246	yHB244 (GAL-HO, Δ*ddc2*) [pNML1: *Myc-DDC2-URA3-CEN*]	This study
yHB247	yHB244 (GAL-HO, Δ*ddc2*) [pHB126: *Myc-ddc2ΔN-URA3-CEN*]	This study
yJK8-1	*matΔ can1 ade2 trp1 his3 ura3 leu2 lys5 cyh2 ade3*::*GalHO adh4*::*HO site*::*HIS3 DDC2-GFP*::*KanR*	36
yHB255	yJK8-1 *ddc2ΔN-GFP* [pBAD79: *RNR3-TRP1-CEN*]	This study

transcription/translation (Promega) and added to recombinant His-RPA or His-RPA-ssDNA beads in binding buffer, and RPA was isolated using His-Select (Sigma Aldrich) or ssDNA-Sepharose beads. Proteins bound to beads were washed with binding buffer three times, eluted, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) prior to blotting.

Kinase assays. ATR kinase assays were performed essentially as described previously (28) with the following alterations. HA-ATRIP and Flag-ATR expression vectors were transfected into 293T cells and the ATR-ATRIP complexes purified by immunoprecipitation. Kinase reactions were performed with the antibody-linked ATR-ATRIP complex. Recombinant TopBP1 and RPA heterotrimer was purified from *E. coli*. The Phas1 substrate was purchased from A.G. Scientific. The in situ Rad53 autophosphorylation assay following denaturation/renaturation was performed as previously described (39).

Cell culture. All cells were grown in Dulbecco modified Eagle medium (DMEM)–7.5% fetal bovine serum. Plasmid transfections were performed with Lipofectamine 2000 (Invitrogen). The ATRIP wild-type (WT) and ATRIPΔN 293T cells were generated by retroviral infection and selection (3). ATRIP small interfering RNA (siRNA) transfections and immunofluorescence methods were also performed as described previously (3).

Protein purification and antibodies. Tandem affinity purification (TAP)-Rfa1 was purified from soluble yeast extracts as described previously (1, 17). RPA70N was kindly provided by Cheryl Arrowsmith in a pET15b vector (Novagen) and was grown on defined M9 medium supplemented with ^{15}N-NH_4Cl and D-glucose and purified over nickel-nitrilotriacetic acid. γH2AX (Upstate), Myc9E10 (Covance), and HA.11 (Covance) were purchased from the indicated companies. Rad53 antibody was a gift from Stephen Elledge. Rfa1 antibody was a gift from Steven Brill.

NMR analysis and homology modeling. Nuclear magnetic resonance (NMR) spectra were collected on ~100 μM ^{15}N-RPA70N in a buffer containing 2 mM β-mercaptoethanol, 50 mM NaCl, and 20 mM Tris–d_{11} at pH 7.4. ATRIP1-107 and ATRIP54-70 were added at a four- to sixfold molar excess to maximize the bound-state population of the observed component, RPA70N. NMR experiments were performed at 25°C using Bruker AVANCE 500 MHz or 600 MHz NMR spectrometers equipped with a 5 mm single axis *z*-gradient Cryoprobe. Two-dimensional, gradient-enhanced ^{15}N-^{1}H heteronuclear single-quantum correlation (HSQC) spectra were recorded with 1,024 complex datum points in the ^{1}H dimension and 96 complex points in ^{15}N dimension. ^{1}H and ^{15}N backbone NMR assignments for RPA70N were kindly provided by Cheryl Arrowsmith.

Chromatin immunoprecipitation (ChIP) analysis. HO expression in strains yHB245 (vector), yHB246 (Myc-DDC2), and yHB247 (Myc-Ddc2ΔN) containing galactose-inducible expression of HO endonuclease was performed as described previously (30). Cells were cross-linked with 1% formaldehyde, lysed, and sonicated to generate DNAs with an average size of 500 bp. Myc-Ddc2 protein-DNA complexes were isolated using Myc9E10 antibody and protein G-Sepharose beads, washed extensively, and eluted from beads. Cross-links were reversed by overnight incubation at 65°C. DNA was precipitated and amplified using the following primers specific to a region adjacent to the HO break site (HO-A or HO-B) or to a region of SMC2: HO-A1 (5′-CTCATCTGTGATTTGTGG-3′), HO-A2 (5′-AGAGGGTCACAGCACTAATACA-3′), HO-B1 (5′-CCAGATTTGTATTAGACGAGGGACGGAGTGA-3′), HO-B2 (5′-AGAGGGTCACAGCACTAAATACAGCTCGAAT-3′), SMC2-1 (5′-AAGAGAAACTTTAGTCAAAACATGGG-3′), and SMC2-2 (5′-CCATCACATTATACTAACTACGG-3′).

RESULTS

Characterization of ATRIP and RPA binding domains. Our analysis started with an examination of the binding of ATRIP fragments to RPA in the absence and presence of ssDNA. Previous analysis of ATRIP identified at least three domains: an N-terminal RPA-ssDNA binding domain, a dimerization domain predicted to fold into a coiled-coil structure, and a C-terminal ATR-interaction domain (2, 3). HA-tagged, intact ATRIP and ATRIP fragments spanning the various domains were generated using a coupled transcription/translation system. These ATRIP proteins were added to purified His-tagged RPA heterotrimer bound to ssDNA displayed on Sepharose beads or His-tagged RPA heterotrimer bound to nickel beads. After incubation and washing, the bound ATRIP proteins were detected by Western blot analysis. We found that all ATRIP fragments containing the N-terminal 107 amino acids bound well to RPA-ssDNA and His-RPA in the absence of DNA (Fig. 1A and B). Thus, the first 107 amino acids of ATRIP contain a protein-protein interaction domain that mediates binding to the RPA heterotrimer.

HA-ATRIP fragments lacking this *N*-terminal *R*PA *b*inding *d*omain of ATRIP (N-RBD) were deficient in binding RPA-ssDNA and His-RPA (Fig. 1A and B). Long exposures of Western blots did show a small degree of association of these ATRIP fragments with RPA. In fact, all of the protein fragments that we tested, including a fragment of Brca1, bound weakly to RPA-ssDNA (Fig. 1A). These interactions may reflect additional RPA-ssDNA binding domains on ATRIP, as has been previously reported (37).

To determine which subunit of the RPA heterotrimer inter-

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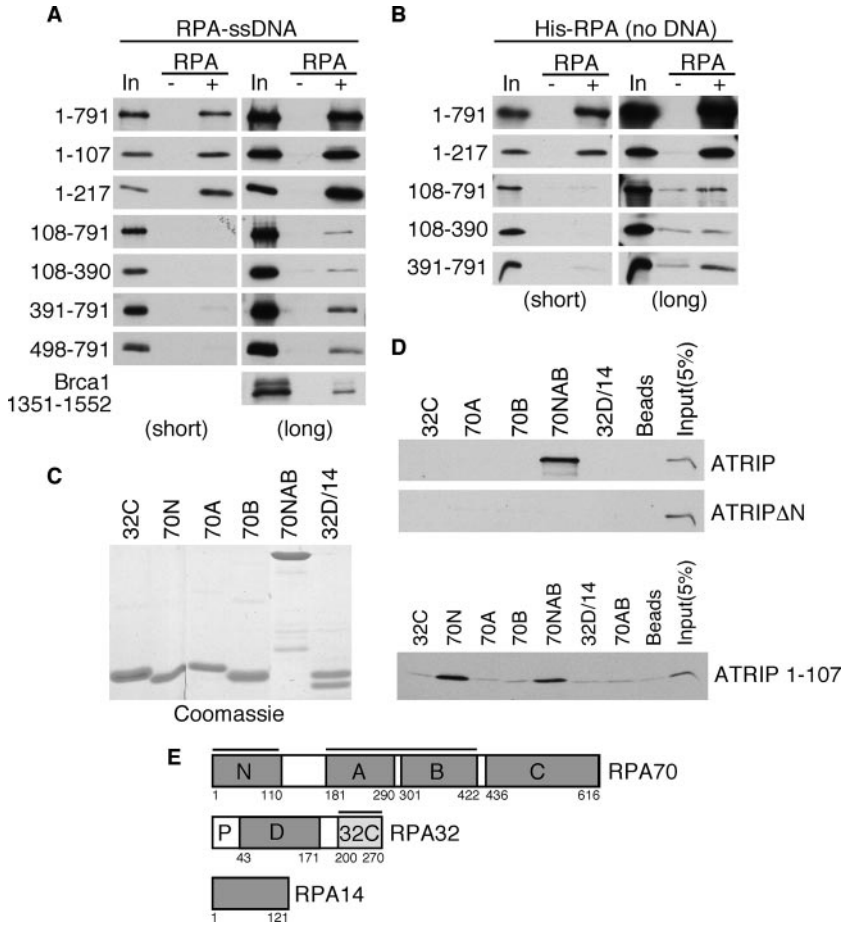

FIG. 1. ATRIP N terminus binds RPA70N in vitro. HA-tagged, full-length ATRIP or ATRIP fragments generated using a coupled transcription/translation system were incubated with single-stranded DNA bound to Sepharose beads in the presence (+) or absence (−) of purified RPA (A) or His-RPA bound to nickel beads (B). After washing, the bound proteins were eluted, separated by SDS-PAGE, blotted, and probed with HA antibody. Input (In) (10%) data are included for comparison. (C and D) Purified, recombinant His-tagged RPA domains were added to in vitro translation reaction mixtures containing HA-ATRIP, HA-ATRIPΔN (C), or HA-ATRIP1-107 (D). Protein complexes were isolated using nickel beads, separated by SDS-PAGE, blotted, and probed using an HA antibody. (E) Diagram of RPA heterotrimer. Black bars above protein segments indicate protein interaction domains.

acts with ATRIP, we purified recombinant RPA domains individually or in combination as His-tagged proteins (Fig. 1C). Using pull-down assays with in vitro-translated ATRIP proteins, we found that full-length HA-ATRIP or the isolated N-RBD bound only to RPA fragments containing the N-terminal RPA70 oligonucleotide/oligosaccharide (OB) fold domain (RPA70N) (Fig. 1D). No significant binding to other RPA domains was detectable, and no binding of ATRIPΔN (ATRIP108-791) protein lacking the N-RBD to any RPA fragment (Fig. 1D) was detectable in this assay. Taken together, these data suggest that the ATRIP N-RBD interacts directly with the 70N domain of RPA.

A conserved acidic domain in the ATRIP N terminus interacts with the basic cleft of the RPA70N OB fold. The specific residues involved in the interaction of RPA and ATRIP were identified using an NMR chemical-shift-mapping approach. This strategy involves monitoring NMR chemical shifts of one protein over the course of a titration with a binding partner. Measurement of the RPA ^{15}N-^{1}H-HSQC NMR spectrum of ^{15}N-enriched RPA70N as ATRIP N-RBD after titration in solution showed that only a subset of the RPA70N signals was affected (Fig. 2A). The observation of effects in the fast-to-intermediate-exchange regimen on the NMR timescale suggests that binding was occurring with a dissociation constant in the low micromolar range. When the chemical shifts are mapped onto the crystal structure of RPA70N (7), it is apparent that ATRIP N-RBD interacts within the basic cleft of RPA70N (Fig. 2B).

Initial insight into characteristics of the RPA70N binding site of the ATRIP N-RBD was obtained from sequence analysis. When the N termini of five ATRIP orthologs were aligned, minimal sequence similarity was observed, with the notable exception of a small, acidic region spanning approximately 15 amino acids (Fig. 2C). On the basis of putative electrostatic complementarity, we hypothesized that this small acidic region made contact with the basic surface in the cleft of the OB fold of RPA70N. To test this hypothesis, an ATRIP peptide spanning the conserved acidic region (ATRIP54-70) was synthesized, and the RPA70N titration was repeated. The titration with the peptide perturbed most of the same residues

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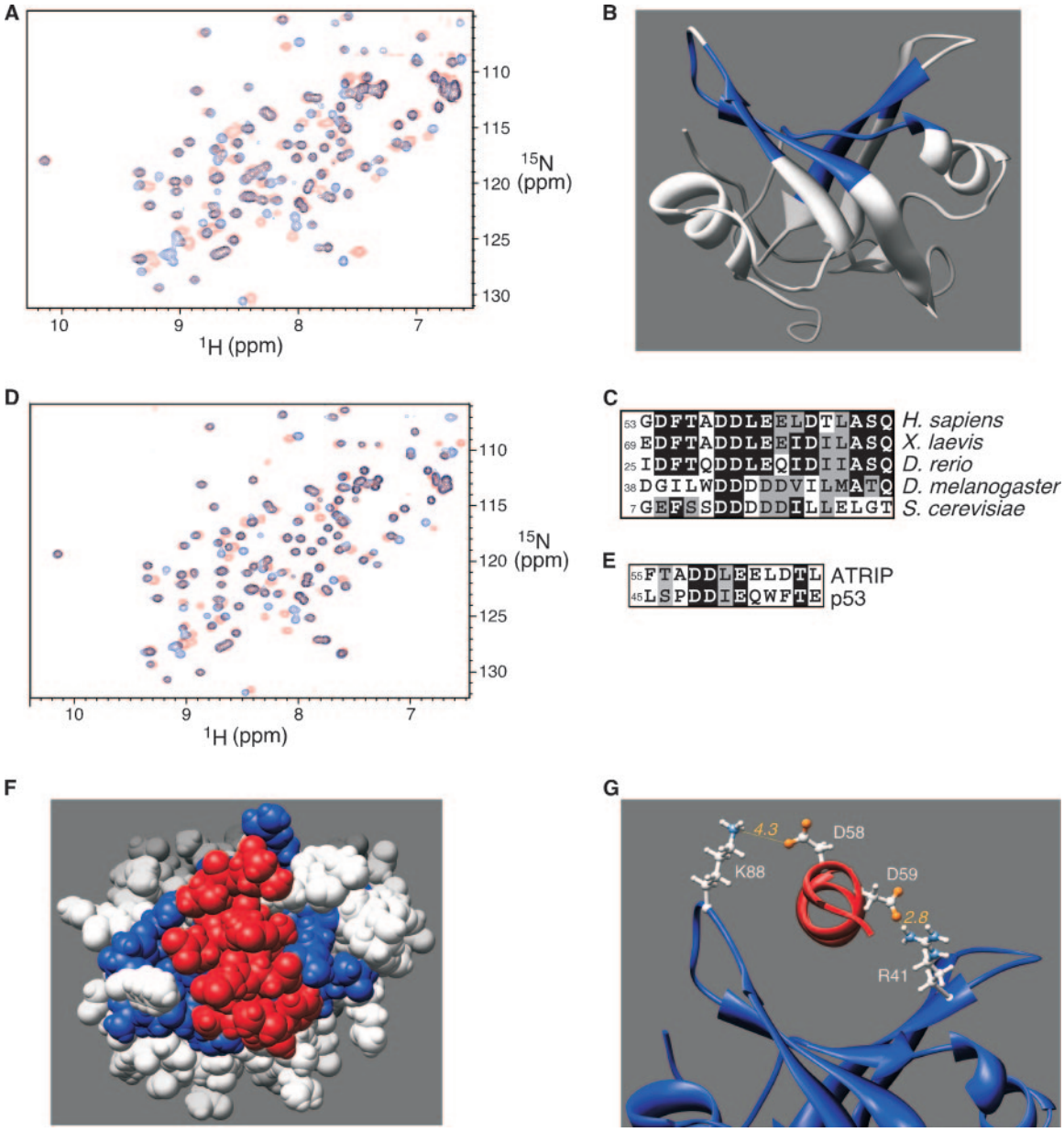

FIG. 2. A conserved acidic domain in the ATRIP N terminus interacts with the basic cleft of RPA70N. (A) The ^{15}N-^{1}H-HSQC NMR spectrum of ^{15}N-enriched-RPA70N in the absence (blue) and presence (red) of ATRIP1-107. (B) RPA70N residues perturbed (blue) upon addition of ATRIP1-107 mapped onto the crystal structure of RPA70N (PDB accession 2B3G). (C) Sequence alignment of the conserved acidic region in the N terminus of five ATRIP orthologues. (D) The ^{15}N-^{1}H-HSQC NMR spectrum of ^{15}N-enriched RPA70N acquired in the absence (blue) and presence (red) of ATRIP54-70. (E) Alignment of the p53 and ATRIP peptides used in homology modeling. (F) Space-filling diagram of RPA70 and ATRIP55-66 (red), with the residues of RPA70N in the ATRIP binding pocket colored blue. (G) Predicted electrostatic interactions between basic RPA70N basic residues K88 and R41 with ATRIP acidic residues D58 and D59.

as the titration with ATRIP N-RBD, indicating that the ATRIP peptide binds in the same manner within the basic cleft of RPA70N (Fig. 2D). In addition, we analyzed the binding of ATRIP1-107 containing charge-reversal mutations at positions D58 and D59 to RPA70N by use of NMR. This mutant binds much more weakly than wild-type ATRIP.

The basic cleft of RPA70N has been shown to bind peptides that can mimic DNA in a manner similar to the binding of ssDNA to the A and B domains of RPA70 (5, 7). RPA70N binds an acidic helical peptide of p53, and the crystal structure of the p53 peptide bound in the cleft was determined by a method previously reported (7). Alignment of this p53 peptide with ATRIP54-70 indicates significant homology between the two peptides (Fig. 2E). Therefore, the crystal structure of RPA70N bound to the p53 peptide was used to generate a homology model for the ATRIP peptide-RPA70N interaction. The strategy involved using the backbone coordinates of the RPA70N and the p53 peptide along with the side chains of

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RPA70N from the crystal structure. The p53 amino acid side chains were replaced with the ATRIP amino acid 55 to 66 side chains, and the best fit of the ATRIP peptide into the constrained RPA70N was determined using ROSETTA (Fig. 2F) (40).

The model predicts that there are several specific electrostatic interactions between the acidic residues on ATRIP and the basic residues on RPA. In particular, the absolutely conserved aspartic acid residues D58 and D59 of ATRIP are likely to make contact with R41 and K88 of RPA70N (Fig. 2G). Basic residues at these positions in RPA are highly conserved. The NMR data and molecular modeling are fully consistent with the previously described pull-down experiment results, indicating that the N terminus of ATRIP binds directly to RPA70N. Importantly, these data create a structural framework within which specific ATRIP-RPA binding mutants can be designed and used for functional analysis.

The N-RBD of human ATRIP is conserved in the *S. cerevisiae* ATRIP ortholog Ddc2. The functional consequences of disrupting the ATRIP-RPA interaction in human cells were previously characterized using an ATRIP mutant lacking the entire N-RBD (ATRIPΔN). Unlike wild-type ATRIP, ATRIPΔN has a severe defect in localizing to damage- or replication stress-induced nuclear foci (3). Despite this localization defect, cells depleted of endogenous ATRIP and complemented with ATRIPΔN exhibit normal ATR-dependent signaling following DNA damage (3). The only checkpoint defect that we have uncovered in the ATRIPΔN-expressing cells is a slight delay in recovery from hydroxyurea (HU)-induced stalling of replication (H. L. Ball, unpublished data). The use of RNA interference in endogenous ATRIP synthesis is not 100% effective, and the results are variable from cell to cell. In addition, the level of retrovirally expressed ATRIP or ATRIPΔN after integration of the retroviral vector is variable and not equivalent to the endogenous protein levels (3). These technical limitations to performing genetic analysis of human cell cultures may confound our ability to detect the phenotypic consequences of abrogating the ATRIP-RPA interaction. Therefore, we sought to examine the physiological role of the ATRIP-RPA interaction in the genetic system of another organism—*S. cerevisiae*.

The RPA binding domain of ATRIP is N terminal to the predicted coiled-coil domain. To determine whether the equivalent region of yeast ATRIP (Ddc2) mediates binding to yeast RPA70 (Rfa1), we deleted 42 amino acids N terminal to the predicted coiled-coil domain of Ddc2 (Ddc2ΔN) (Fig. 3A). Binding of Ddc2 and Ddc2ΔN to Rfa1 was assayed using coimmunoprecipitation. Myc-tagged Ddc2 (WT) or Ddc2ΔN (ΔN) was expressed from plasmids under the control of the endogenous *DDC2* promoter in Δ*ddc2* cells containing HA-tagged Mec1. Myc-Ddc2 and Myc-Ddc2ΔN were immunoprecipitated using a Myc antibody, and the coassociated Rfa1 and Mec1 were assayed by Western blotting. As expected, both Mec1 and Rfa1 were coimmunoprecipitated with Ddc2 (Fig. 3B). In comparison, Rfa1 association with Ddc2ΔN was greatly reduced, although Ddc2ΔN continued to bind Mec1 (Fig. 3B). These data suggest that the N terminus of Ddc2 is required for a stable Ddc2-Rfa1 interaction. The amount of Rfa1-associated Ddc2 was not altered by exposing cells to UV damage, suggesting that the Ddc2-Rfa1 interaction may not be regulated by DNA damage (Fig. 3B). However, these experiments utilized soluble extracts, so it is possible that the interaction with DNA-bound RPA is regulated.

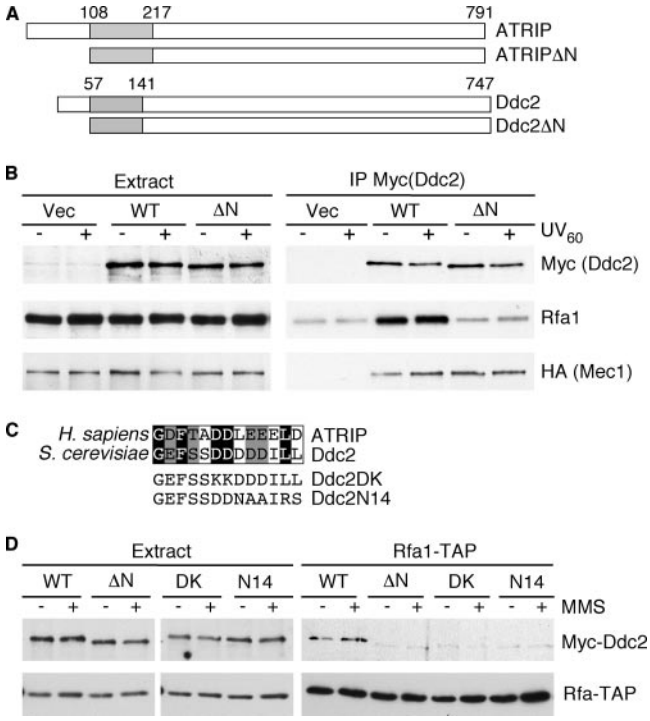

FIG. 3. Ddc2-Rfa1 interaction requires the conserved acidic region of Ddc2. (A) Schematic diagram of ATRIP, RPA-binding mutant ATRIP (ATRIPΔN), Ddc2, and Ddc2ΔN. Locations of predicted coiled-coil domains (gray) are indicated. (B) Δ*ddc2 HA-MEC1* yeast containing myc-vector (Vec), myc-Ddc2 (WT), or myc-Ddc2ΔN (ΔN) were exposed to 0 (−) or 60 (+) J/m^2 UV and harvested 1 h later. Myc immunoprecipitates from soluble extracts were separated by SDS-PAGE, blotted, and probed with Myc, Rfa1, and HA antibodies. (C) Alignment of conserved acidic region in the N terminus of ATRIP and Ddc2 and schematic of mutations generated in this region of Ddc2. (D) Yeasts containing TAP-Rfa1 and Ddc2 (WT), Ddc2ΔN (ΔN), Ddc2DK (DK), or Ddc2N14 (N14) were damaged with 0.01% MMS (+) or left untreated (−) and harvested 1 h later. Cells were lysed, and TAP-Rfa1 was isolated using immunoglobulin G beads. TAP protein complexes were separated by SDS-PAGE and Western blotted using Myc (Ddc2) and Rfa1 antibodies.

Sequence alignment of human and yeast ATRIP indicates that the small acidic region in the ATRIP N-RBD is conserved (Fig. 2C). The homology model generated from NMR data predicts that the absolutely conserved aspartic acid residues in this region (D12 and D13 in Ddc2) could make contacts with conserved basic amino acids on yeast Rfa1. Therefore, we hypothesized that mutating these residues would disrupt Ddc2-Rfa1 binding. To test this hypothesis a Ddc2 mutant was generated with aspartic acid-to-lysine charge-reversal mutations in these two aspartic acids (Ddc2DK) (Fig. 3C). In addition, a mutant (Ddc2N14) was generated replacing Ddc2 residues 14 to 19 with a peptide (NAAIRS) that is known to adopt a helical conformation (Fig. 3C). Myc-Ddc2, Myc-Ddc2ΔN, Myc-Ddc2DK, or Myc-Ddc2N14 was expressed in a yeast strain containing TAP-tagged Rfa1. TAP purification of Rfa1 protein complexes indicated they contained Myc-Ddc2 whether or not cells were pretreated with methyl methanesulfonate (MMS)

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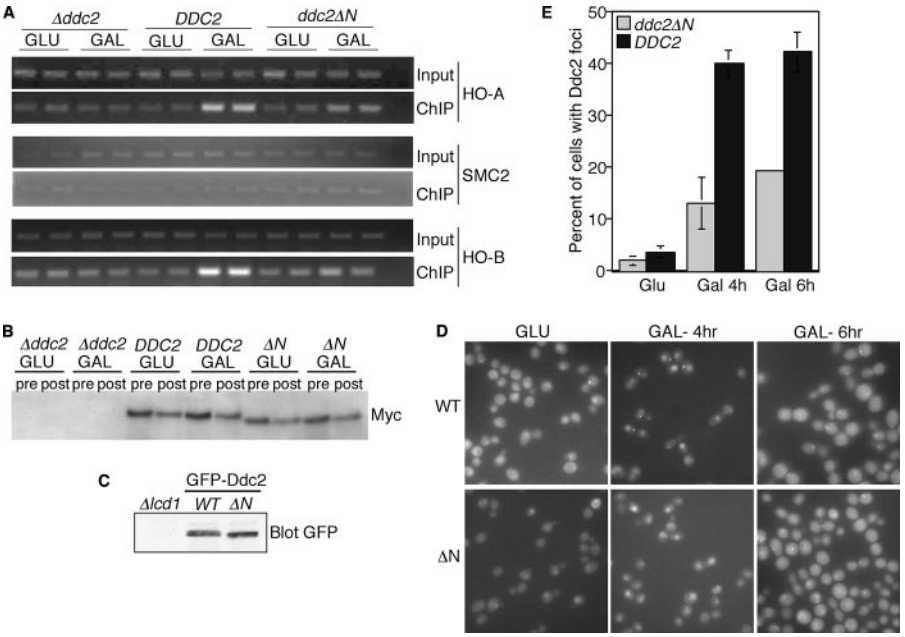

FIG. 4. Ddc2 lacking the N-terminal Rfa1 binding domain is defective in localizing to sites of DNA damage. (A) Δ*ddc2* yeasts transformed with a centromeric plasmid expressing Myc-Ddc2 or Myc-Ddc2ΔN from the *DDC2* promoter and harboring a galactose-inducible HO endonuclease were grown to log phase in raffinose-containing media. Galactose (GAL) or glucose (GLU) was added to induce or suppress HO-endonuclease expression. One hour after sugar addition, cells were cross-linked using formaldehyde and harvested. Cells were lysed and sonicated, and Myc-Ddc2 proteins were immunoprecipitated with a myc antibody. Cross-links were reversed, and associated DNA sequences were amplified by PCR using primers specific to regions adjacent to the HO break site (HO-A, HO-B) or to the SMC2 gene (SMC2). Samples were prepared in duplicate. Input samples represent 5% of input into immunoprecipitation reactions. (B) Equal volumes of immunoprecipitation reaction mixtures before (pre) or after (post) isolation of Myc-Ddc2 proteins were separated by SDS-PAGE, blotted, and probed with a myc antibody. (C) Extracts from Δ*ddc2* yeast, or from yeast expressing GFP-Ddc2 (WT) or GFP-Ddc2ΔN (ΔN), were separated by SDS-PAGE and Western blotted using a GFP antibody. (D) Yeasts expressing GFP-Ddc2 (WT) or GFP-Ddc2ΔN (ΔN) and galactose-inducible HO endonuclease were grown to log phase in liquid culture. Glucose (GLU) or galactose (GAL) was added to suppress or induce DNA double-strand-break formation. GFP fluorescence was visualized on a Zeiss Axioplan fluorescent microscope. (E) Quantitation of HO-induced focus formation of GFP-Ddc2 or GFP-Ddc2ΔN 4 h or 6 h after induction of HO endonuclease expression. Error bars represent standard deviations of the results from three experiments.

(Fig. 3D). In contrast, TAP-Rfa1 purifications contained minimal Myc-Ddc2ΔN, Myc-Ddc2DK, or Myc-Ddc2N14 protein (Fig. 3C). These results confirm that the conserved acidic region in the N terminus of Ddc2 is required for a stable Ddc2-Rfa1 association.

Ddc2-Rfa1 interaction is required for localization of Ddc2 to sites of DNA damage. Human ATRIP lacking the N-terminal RPA binding domain (ATRIPΔN) is defective in DNA-damage-induced focus formation (3). To determine whether the interaction between the N terminus of Ddc2 and Rfa1 is also required for the localization of Ddc2 to sites of DNA damage we assayed Ddc2 localization by use of ChIP analysis and focus formation. To do this we used the inducible HO nuclease system, which introduces a single double-strand break in the yeast genome (29). The induction of a double-strand break in Δ*ddc2*, *DDC2*, or *ddc2ΔN* yeast strains harboring a galactose-inducible HO endonuclease was diagnosed by comparing PCR products generated using primer sets adjacent to or spanning the HO cleavage site, and the results were equal in all strains. One hour after HO induction, cells were treated with cross-linking agent, lysed, and sonicated, and Myc-Ddc2 protein-DNA complexes were isolated by immunoprecipitation. Myc-Ddc2-bound DNA fragments were recovered and amplified by PCR using two different HO primer sets (HO-A and HO-B) adjacent to the HO cleavage site. As a control we amplified a region of the *SMC2* gene that is on a chromosome different than that with the HO cleavage site. WT Ddc2 specifically accumulated at the HO cleavage site but not at the SMC2 site after induction of the HO endonuclease (Fig. 4A). Compared to wild-type Ddc2 results, the accumulation of Ddc2ΔN at the HO break site was severely reduced although not completely abrogated (Fig. 4A). Quantitation of the results of ChIP experiments indicated that Ddc2 binding to the HO cleavage site is fivefold greater than Ddc2ΔN binding. Ddc2 and Ddc2ΔN were expressed at equal levels, and the levels of efficiency of immunoprecipitation were equal in all samples (Fig. 4B).

To determine whether the defect in Ddc2ΔN accumulation at sites of DNA double-strand breaks as detected using ChIP correlated with a defect in accumulation of Ddc2ΔN into DNA-damage-induced nuclear foci we fused a C-terminal green fluorescent protein (GFP) tag onto Ddc2 and Ddc2ΔN. Equal expression of GFP-Ddc2 and GFP-Ddc2ΔN was assessed by Western blotting with an antibody specific to the GFP tag (Fig. 4C). HO-endonuclease expression in *GFP-DDC2* and *GFP-ddc2ΔN* strains was induced and Ddc2 localization monitored by fluorescence microscopy. Induction of a DNA break caused GFP-Ddc2 to accumulate into one distinct

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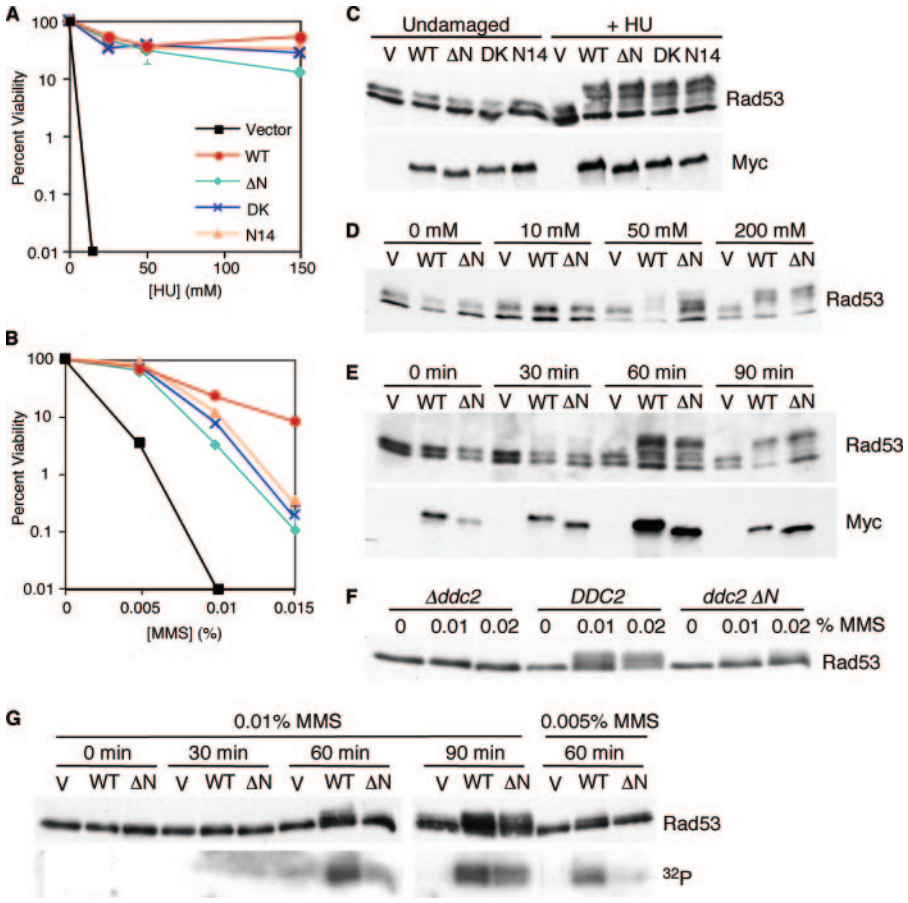

FIG. 5. Disruption of Ddc2-Rfa1 interaction impairs the DNA damage response. (A and B) Yeasts lacking Ddc2 (Vector) or expressing Ddc2 (WT), Ddc2ΔN (ΔN), Ddc2DK (DK), or Ddc2N14 (N14) were grown to log phase in liquid culture and plated onto rich media containing increasing amounts of HU (A) or MMS (B). Percent viability was calculated as the number of colonies surviving at each dose compared to the number of colonies that survived on plates lacking HU or MMS. Data represent the averages of the results of three experiments. Standard deviations were smaller than symbol width in most cases. (C to E) Yeast strains were arrested in G_1 with alpha factor and released into rich media in the presence of 200 mM HU (C and E) or in the indicated concentration of HU (D). Cells were harvested 1 h (C and D) or at the indicated times (E) after G_1 release, and trichloroacetic acid was precipitated. Lysates were separated by SDS-PAGE, blotted, and probed with Rad53 or Myc antibodies. (F and G) Δ*ddc2* (V), *DDC2* (WT), or *ddc2ΔN* (ΔN) yeasts were grown to log phase in liquid culture, arrested in G_1 with alpha factor, and released into media containing the indicated doses of MMS and harvested 1 h post G_1 release (F) or at the indicated various time points after G_1 release (G). Cells were lysed, and proteins were separated by SDS-PAGE, blotted, and probed with Rad53 antibody. (G) Membranes containing immobilized proteins were subjected to in situ autophosphorylation to assay Rad53 autophosphorylation activity.

focus per cell in 40% and 42% of the cells at 4 h and 6 h after HO induction, respectively (Fig. 4D and E). Unlike GFP-Ddc2, GFP-Ddc2ΔN formed a focus in only 13% and 19% of cells after 4 h and 6 h of HO induction, respectively (Fig. 4D and E). Additionally, in cells that did demonstrate HO-induced GFP-Ddc2ΔN foci, the foci were noticeably smaller than GFP-Ddc2 foci (Fig. 4D). Taken together, these data demonstrate that, consistent with the role of ATRIP-RPA interaction in human cells, Ddc2-Rfa1 interaction is required for efficient localization of Ddc2 to sites of DNA damage. Since the N-RBD of both ATRIP and Ddc2 is required for recruitment of the ATR-ATRIP/Mec1-Ddc2 checkpoint complexes to DNA lesions, we have named this domain the *c*heckpoint protein *r*ecruitment *d*omain (CRD).

Disruption of the Ddc2-Rfa1 interaction sensitizes cells to DNA damage. To examine the function of the Ddc2-Rfa1 interaction in Mec1-dependent checkpoint signaling, we first determined whether disrupting binding sensitized cells to replication stress or DNA damage. Δ*ddc2* yeasts expressing Ddc2, Ddc2ΔN, Ddc2DK, or Ddc2N14 were grown to log phase in liquid culture and plated onto media containing increasing amounts of HU or MMS. Yeasts lacking Ddc2 altogether are extremely sensitive to even low doses of HU or MMS (Fig. 5A and B). In contrast, none of the mutant *ddc2* strains were sensitive to low doses of HU, and only a very small difference was visible compared to the *DDC2* strain results at the highest HU concentration (Fig. 5A). The difference in sensitivity to genotoxic agents between WT and mutant strains was more apparent in response to the MMS. *ddc2ΔN*, *ddc2DK*, and *ddc2N14* strains were more sensitive to high doses of MMS than the *DDC2* strain but much less sensitive than Δ*ddc2* yeast. For example, at a dose of 0.01% MMS, *ddc2ΔN* cell viability was reduced to 3% compared to 23% for *DDC2* and less than 0.01% for Δ*ddc2* yeast (Fig. 5B). At 0.15% MMS there was a difference of an order of magnitude in the viability of *ddc2ΔN ddc2DK* and *ddc2N14* strains compared to WT *DDC2* results (Fig. 5B). These results suggest that the Ddc2 CRD is

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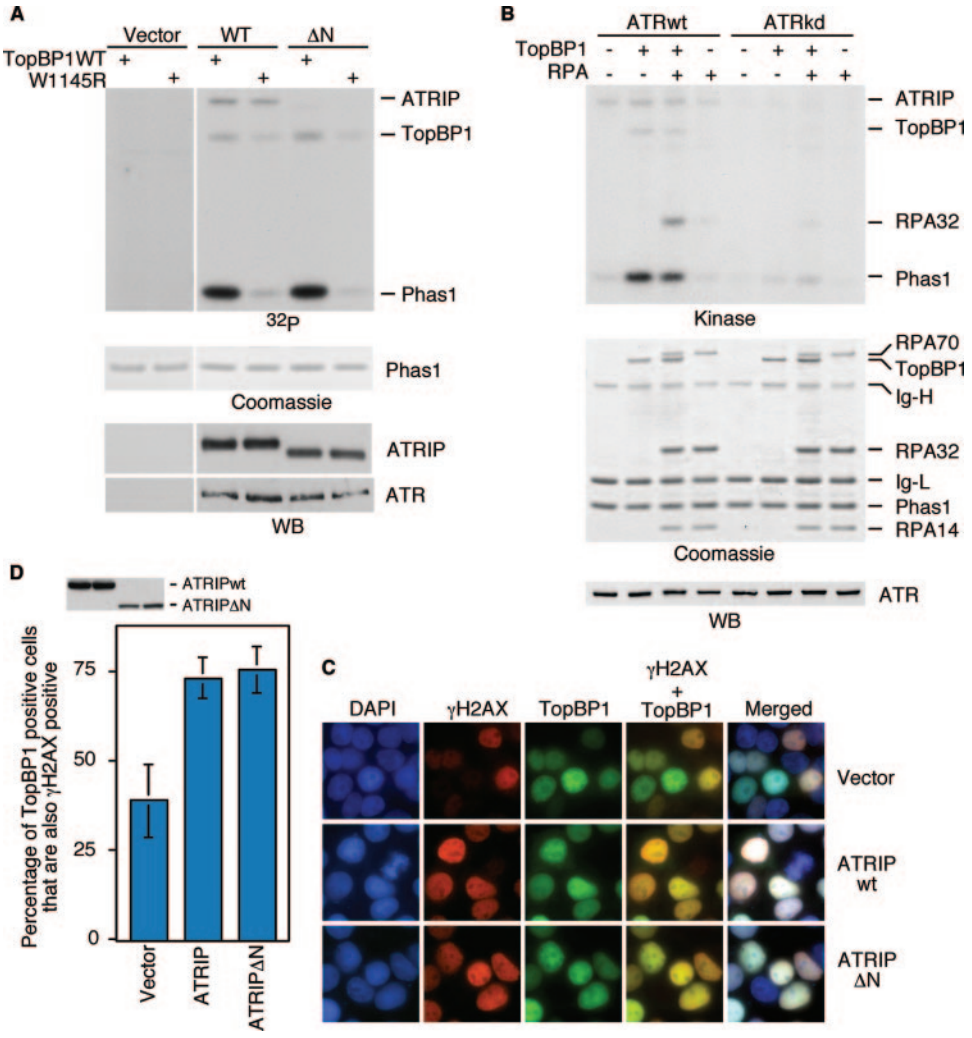

FIG. 6. TopBP1 activates ATR-ATRIP complexes independently of RPA. (A) Wild-type ATR-ATRIP or ATR-ATRIPΔN complexes were isolated from transfected 293T cells and incubated with recombinant wild-type TopBP1 978–1286 (WT) or TopBP1 978–1286 W1145R (WR), Phas1 substrate, and [γ-^{32}P]ATP. Kinase reaction mixtures were separated by SDS-PAGE, stained with Coomassie blue, and exposed to film (^{32}P). A duplicate gel was blotted and probed with anti-ATRIP and anti-ATR antibodies (WB). (B) Wild-type ATR-ATRIP or kinase-dead ATR-ATRIP immune complexes were isolated from transfected 293T cells and incubated with recombinant TopBP1 and/or RPA heterotrimer in the presence of Phas1 substrate and [γ-^{32}P]ATP. Kinase reaction mixtures were separated by SDS-PAGE, stained with Coomassie blue or blotted, and exposed to film (^{32}P) or probed with anti-ATR antibodies (WB). (C and D) 293T cells stably expressing siRNA-resistant ATRIP, ATRIPΔN, or empty vector control were transfected with ATRIP siRNA to deplete endogenous ATRIP. Two days after siRNA transfection, the cells were transfected with GFP-TopB1 978–1286 expression construct. Twenty-four hours later the cells were fixed and stained with antibodies to γH2AX. (C) Representative images collected on a Zeiss Axioplan microscope with the same exposure times. (D) Quantitation of the percentages of the GFP-TopBP1-expressing cells that contained phosphorylated H2AX. Error bars represent standard deviations. The inset presents a Western blot showing the relative expression levels of ATRIP and ATRIPΔN.

important for survival of cells following exposure to the DNA-alkylating agent MMS.

To directly examine the role of the Ddc2 CRD in checkpoint signaling, we tested the ability of wild-type Ddc2 or Ddc2 mutants to support Mec1-dependent Rad53 phosphorylation. Yeast were grown to log phase, arrested in G_1 with alpha factor, released in the presence or absence of 200 mM HU, and harvested at various time points after release. Cell lysates were generated, and proteins were separated by SDS-PAGE and blotted using antibodies to Rad53. Rad53 phosphorylation is detectable by an electrophoretic mobility shift and is defective in Δ*ddc2* yeast, as seen by the absence of a slower-migrating form of Rad53 (Fig. 5C). Consistent with the lack of HU sensitivity, Ddc2-Rfa binding mutants Ddc2ΔN, Ddc2DK, and Ddc2N14 all support Rad53 phosphorylation after exposure to HU as efficiently as Ddc2 (Fig. 5C). Detailed time course and dose-response experiments also failed to detect a significant Rad53 activation defect in the Rfa1-binding mutant strains in response to HU (Fig. 5D and E). These results are consistent with the effects of equivalent mutations in human ATRIP which fail to disrupt ATR signaling in response to HU (3).

In contrast, we did observe an attenuation of Mec1 signaling in these yeast strains in response to the presence of MMS. Strains were grown to log phase, arrested in G_1 with alpha

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factor, and released into media containing various doses of MMS. Phosphorylated Rad53 is visible in *DDC2* cells after the addition of 0.01% MMS (Fig. 5F). However, Rad53 phosphorylation is attenuated in *ddc2ΔN* cells, indicating that optimal Rad53 phosphorylation after exposure to MMS depends upon Ddc2-Rfa1 binding (Fig. 5F). The defect in Mec1 signaling after MMS treatment was most apparent at early time points after release into S phase (Fig. 5G). For example, at the 60-min time point in the presence of either 0.005% or 0.01% MMS both the phosphorylation-dependent shift of Rad53 and Rad53 kinase activity are significantly reduced in the *ddc2ΔN* strain compared to *DDC2* results (Fig. 5G). However, at later time points (90 min), cells expressing Ddc2ΔN showed considerable Rad53 activation whereas Δ*ddc2* cells did not (Fig. 5G). These defects at early time points were not due to a difference in the results of release of yeast from alpha factor arrest, since all strains released equivalently. Taken together, these results suggest that Ddc2-Rfa1 binding and localization to damage sites is required for optimal checkpoint activation after exposure to MMS.

TopBP1-dependent ATR activation can occur independently of RPA. TopBP1 was recently shown to bind and activate ATR (28). This activation activity was localized to a small fragment of TopBP1 between two BRCT repeat domains. These authors also found that TopBP1 binding and activation of xATR requires xATRIP. We confirmed that TopBP1 activates ATR-ATRIP complexes in an ATRIP-dependent manner (Fig. 6 and data not shown). To determine whether the ATRIP CRD influences TopBP1 activation of ATR, we purified either wild-type ATR-ATRIP complexes or ATR-ATRIPΔN complexes. Addition of the TopBP1 fragment but not of an equivalent fragment containing an inactivating mutation (W1145R) to ATR-ATRIP complexes stimulated ATR activity toward substrates in an immune complex kinase reaction (Fig. 6A). Activation of the ATR-ATRIPΔN complex upon the addition of TopBP1 was equal to the activation of ATR-ATRIP (Fig. 6A). These findings are consistent with those of Kumagai et al., who found that xTopBP1 stimulates activation of xATR-xATRIP complexes containing a xATRIP protein lacking the N terminus (28). Therefore, TopBP1-dependent ATR activation does not require the ATRIP CRD.

We next assayed whether RPA or RPA-ssDNA influences ATR activity or TopBP1-dependent ATR activation. Addition of TopBP1 to ATR-ATRIP stimulated ATR kinase activity (Fig. 6B). In contrast, addition of RPA (data not shown) or RPA-ssDNA to ATR kinase assays failed to stimulate ATR activity (Fig. 6B). RPA-ssDNA also had no influence on TopBP1 activation of ATR (Fig. 6B). RPA32 phosphorylation by ATR is stimulated by TopBP1. In addition, we also observed significant phosphorylation of the TopBP1 fragment, ATRIP, and ATR in these experiments. However, in contrast to the results seen with other proteins added to the kinase assay, the amount of autophosphorylation on the ATR-ATRIP complex was not altered significantly by the addition of the TopBP1 fragment. These results suggest that RPA-ssDNA binding to ATR-ATRIP does not influence the kinase activity of ATR. Furthermore, the function of ATRIP required to promote TopBP1-dependent activation of ATR can be separated from its RPA binding activity. However, the results do not exclude the possibility that specific RPA-DNA structures found in cells might regulate kinase activity.

To confirm these results in cells, a GFP-TopBP1 fragment containing the region that activates ATR was transfected into human cells. The cells were engineered to stably express siRNA-resistant wild-type ATRIP, ATRIPΔN, or an empty vector and were transfected with the ATRIP siRNA prior to GFP-TopBP1 transfection. Depletion of endogenous ATRIP by siRNA transfection in these cells is approximately 80% (3). Twenty-four hours after transfection of GFP-TopBP1, cells were fixed and stained for a marker of ATR activation (γH2AX). Overexpression of GFP-TopBP1 in cells containing wild-type ATRIP or ATRIPΔN caused phosphorylation of H2AX throughout the chromatin (not in distinct foci, as would be observable in response to a DNA-damaging agent) (Fig. 6C). However, both the intensity of phosphorylation and the number of cells containing phosphorylated H2AX were greatly reduced in cells depleted of ATRIP, indicating that this result was due to ATR-ATRIP signaling (Fig. 6C and D). These results confirm that TopBP1 can activate ATR in cells when highly overexpressed even when ATR-ATRIP complexes lack the RPA binding domain and fail to localize to specific sites of DNA damage or replication stress. The overexpression of the TopBP1 fragment likely bypasses the regulation of TopBP1-dependent ATR activation that exists under physiological conditions.

DISCUSSION

A checkpoint protein recruitment domain (CRD) has been identified in the N terminus of ATRIP and Ddc2. This domain binds directly to RPA70N, recruits ATR-ATRIP/Mec1-Ddc2 complexes to sites of DNA damage, and promotes ATR-dependent checkpoint signaling in response to MMS. These findings are consistent with those of Kim et al., who reported that an N-terminal domain of *Xenopus* ATRIP is required for binding to RPA (25). RPA is a modular protein, and it often makes more than one contact with its interacting partners. Indeed, Namiki and Zou identified three large regions of ATRIP that may interact with RPA-ssDNA (37). Since no functional data were reported, additional experiments will be required to define and study the function of any other ATRIP surfaces that make direct contacts with RPA subunits. However, our data indicate that the N-terminal CRD domains of ATRIP and Ddc2 are required for the stable binding of ATRIP/Ddc2 to RPA and are necessary for retention of ATR-ATRIP/Mec1-Ddc2 at sites of DNA damage in cells.

A model of the interaction of RPA70N with a conserved ATRIP peptide within the CRD was generated using NMR data and molecular modeling from the crystal structure of a p53 peptide bound to RPA70N. The model predicts that acidic ATRIP residues (D58 and D59) make direct contacts with basic RPA70N residues (R41 and K88) in the basic cleft of the RPA70N OB fold domain. All of these amino acids are highly conserved. As predicted by this model, mutations reversing the charges on the equivalent aspartic acid residues in Ddc2 (D12K and D13K) abrogate binding to Rfa1. Interestingly, the well-characterized *rfa1-t11* mutant, which is known to be replication competent but DNA-damage-response deficient, contains a single charge-reversal mutation at K45, the residue

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equivalent to R41 in human RPA (49). Indeed, as our model would predict, *rfa-t11* is deficient in recruiting Ddc2 to double-strand breaks (23, 52) and in binding Ddc2 (H. L. Ball, unpublished data). The *rfa-t11* mutant is also recombination deficient, suggesting that this basic cleft in RPA70N may be a key ligand in DNA damage responses (44, 49). It will be interesting to determine whether other DNA damage response proteins also contain acidic helices that bind within this cleft of RPA70N. It is also noteworthy that an ATR phosphorylation site (S68) is located within the ATRIP CRD just downstream of the acidic peptide that binds to the RPA basic cleft (21). Moreover, RPA70N appears to interact with the RPA32 N terminus when it is phosphorylated by checkpoint kinases (6). Therefore, phosphorylation of either ATRIP or RPA may be a means to regulate the ATR-RPA interaction.

The phenotypic consequences of disrupting the ATRIP CRD-RPA70 interaction are similar in human and yeast cells. In contrast to ATRIP or Ddc2 loss of function, cells containing mutations that disrupt the CRD are only mildly sensitive to DNA-damaging agents and partially compromised in checkpoint signaling. In fact, the response to HU is nearly indistinguishable from wild-type results despite severe defects in ATR-ATRIP/Mec1-Ddc2 localization. Functions of ATRIP in addition to RPA binding are also critical for ATR signaling. These functions include oligomerization (2, 20), ATR stabilization (12), and an undefined activity important for TopBP1-dependent activation of ATR.

The reason for the increased sensitivity of Ddc2 lacking the CRD to damage that generates DNA adducts (MMS) compared to depletion of nucleotides (HU) is unknown. Both types of genotoxic stress activate Mec1 during replication and stall replication forks (48). One potential explanation for this difference may be the amount of RPA-ssDNA present at various types of DNA lesions. Mec1-Ddc2ΔN complexes may have some residual association with Rfa1 and can still partially localize to double-strand breaks. Perhaps there is more RPA-ssDNA at an HU-stalled fork than at an MMS-induced lesion, increasing the requirement for the Ddc2 CRD at the MMS lesion. Alternatively, the recruitment and activation mechanisms of ATR-ATRIP and Mec1-Ddc2 at MMS or HU lesions may be different. Accumulating evidence suggests that additional protein-protein and protein-DNA interactions other than the ATRIP-RPA interaction may help recruit ATR-ATRIP to DNA lesions (8, 50). Also, Ddc2 contains a DNA end-binding activity localized to a region C terminal to the predicted coiled-coil domain (42). Perhaps these alternative modes of ATR-ATRIP/Mec1-Ddc2 recruitment function differently at HU and MMS lesions.

Consistent with the report by Kumagai and coworkers, we have found that TopBP1 activates ATR and that TopBP1-dependent activation of ATR is ATRIP dependent and does not require the ATRIP CRD (28). RPA-ssDNA, in contrast, does not stimulate ATR kinase activity in immune complex in vitro kinase reactions, and TopBP1-dependent activation of ATR is not altered by adding RPA or RPA-ssDNA to the kinase reaction. These results suggest that TopBP1-dependent ATR activation can be separated from ATRIP-RPA binding. The affinity of TopBP1 for ATR-ATRIP is weak and difficult to detect by coimmunoprecipitations (28). The accumulation of ATR-ATRIP and TopBP1 at sites of damage may facilitate this low-affinity interaction by increasing the local concentration of these proteins.

Taken together, these data support a multistep model proposed by Dunphy and colleagues (27) for the activation of ATR checkpoint signaling. ATR recruitment to sites of DNA damage and replication stress occurs in part through a direct interaction between the ATRIP CRD and RPA70N. TopBP1 is recruited independently through an interaction with Rad9 (16, 18, 35, 46). The assembly of ATR-ATRIP and TopBP1 at the lesion facilitates TopBP1-dependent ATR activation and, in turn, phosphorylation of ATR substrates. Accessory proteins such as claspin are also required for phosphorylation of specific substrates (26, 31). Localization may also serve to bring ATR to the vicinity of key substrates involved in fork stabilization or other aspects of checkpoint regulation. Within this model, ATRIP is a key ATR regulator since it promotes both the localization and activation of ATR. The model suggests that ATR localization to a damage site precedes its activation. However, it remains possible that ATR can be activated without localization. Indeed, when TopBP1 is highly overexpressed it activates ATR throughout the nucleus in the absence of a DNA lesion (Fig. 6C). Furthermore, stable retention of ATR at a damage site is not required for ATR activation, at least, not in response to relatively high doses of UV (3). Thus, further experiments are required to definitively determine whether localization must precede activation.

ATR, ATM, and other checkpoint signaling pathways are activated by many cancer therapies and regulate the cellular outcomes of these treatments. Disruption of ATR, ATM, and DNA-PK kinases sensitizes cells to radiation and chemotherapy. Since mutations in DNA repair and DNA-damage response pathways are common in cancer cells, these cells are particularly sensitive to disruption of additional pathways. This rationale has driven the development of small molecule inhibitors of DNA damage-responsive kinases for use as chemo- or radio-sensitizing agents (22, 32). Thus far, specific inhibitors of ATM and DNA-PK kinases have been developed, built around competitive inhibition by binding of ATP analogues (19). However, specific inhibitors of the ATR kinase have not been found. Unique properties of ATR, such as its requirement for ATRIP, may provide an alternative means of disrupting ATR signaling. Hence, the molecular characterization of ATRIP structure and function as described here may provide a useful starting point for the development of an ATR-targeted therapy.

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TopBP1 activates ATR through ATRIP and a PIKK regulatory domain

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The ATR (ATM and Rad3-related) kinase and its regulatory partner ATRIP (ATR-interacting protein) coordinate checkpoint responses to DNA damage and replication stress. TopBP1 functions as a general activator of ATR. However, the mechanism by which TopBP1 activates ATR is unknown. Here, we show that ATRIP contains a TopBP1-interacting region that is necessary for the association of TopBP1 and ATR, for TopBP1-mediated activation of ATR, and for cells to survive and recover DNA synthesis following replication stress. We demonstrate that this region is functionally conserved in the *Saccharomyces cerevisiae* ATRIP ortholog Ddc2, suggesting a conserved mechanism of regulation. In addition, we identify a domain of ATR that is critical for its activation by TopBP1. Mutations of the ATR PRD (PIKK [phosphoinositide 3-kinase related kinase] Regulatory Domain) do not affect the basal kinase activity of ATR but prevent its activation. Cellular complementation experiments demonstrate that TopBP1-mediated ATR activation is required for checkpoint signaling and cellular viability. The PRDs of ATM and mTOR (mammalian target of rapamycin) were shown previously to regulate the activities of these kinases, and our data indicate that the DNA-PKcs (DNA-dependent protein kinase catalytic subunit) PRD is important for DNA-PKcs regulation. Therefore, divergent amino acid sequences within the PRD and a unique protein partner allow each of these PIK kinases to respond to distinct cellular events.

[*Keywords*: ATR; ATRIP; TopBP1; checkpoint; PIKK; Ddc2]

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The DNA damage response maintains genome integrity through the coordination of DNA replication, cell cycle progression, transcription, apoptosis, senescence, and DNA repair. At the apex of this pathway are two closely related kinases—ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related)—that phosphorylate hundreds of proteins, including the tumor suppressor proteins BRCA1 and p53 (Kastan and Bartek 2004). ATM primarily responds to DNA double-strand breaks, while ATR is activated by replication stress and ssDNA gaps.

ATR and ATM are members of the PIKK (phosphoinositide 3-kinase related kinases) family of protein kinases, which regulate diverse biological activities. Other members of this family include mTOR (mammalian target of rapamycin), which coordinates protein synthesis and cell growth; DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which promotes DNA double-strand break repair by nonhomologous end-joining; and SMG1, which regulates nonsense-mediated mRNA decay (Abraham 2004). PIKKs are large proteins (2549–4128 amino acids) with a common domain architecture. They contain dozens of N-terminal HEAT repeats that may mediate protein–protein interactions and a highly conserved C-terminal kinase domain flanked by the FAT (FRAP, ATM, TRRAP) and FATC (FAT C terminus) domains of unknown function.

Homozygous mutations in *ATM* cause ataxia-telangiectasia, which is characterized by progressive neurodegeneration and severe cancer predisposition, and people with a heterozygous *ATM* mutation have an increased risk of breast cancer (Shiloh 2003). *ATR* mutations are infrequent because ATR is essential for cell viability and embryonic development (Brown and Baltimore 2000; Cortez et al. 2001). However, hypomorphic mutations in *ATR* have been linked to rare cases of Seckel syndrome (O'Driscoll et al. 2003). This syndrome is characterized by microcephaly and developmental defects.

ATR forms a stable complex with ATRIP (ATR-interacting protein), which regulates the localization of ATR and is essential for ATR signaling in response to DNA damage and replication stress (Cortez et al. 2001; Zou and Elledge 2003; Ball et al. 2005). Many proteins in addition to ATRIP participate in the ATR pathway including TopBP1, which functions in both the initiation of DNA replication and checkpoint signaling (Garcia et al. 2005). Depletion of TopBP1 in mammalian cells does not affect the localization of ATR–ATRIP to sites of DNA damage but inhibits the damage-inducible phosphoryla-

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tion of ATR substrates (Liu et al. 2006). As first discovered by Dunphy and colleagues, the TopBP1 protein directly stimulates the kinase activity of the ATR–ATRIP complex in vitro and in cells (Kumagai et al. 2006). A region between the sixth and seventh BRCT domains of TopBP1 called the ATR Activation Domain (AAD) is sufficient to activate ATR (Kumagai et al. 2006). The mechanisms that regulate TopBP1 access to ATR–ATRIP are likely to be complex but include post-translational modifications and recruitment of TopBP1 independently of ATR–ATRIP to sites of replication stress or DNA damage (Delacroix et al. 2007; Lee et al. 2007; Yoo et al. 2007). TopBP1 binds to the phosphorylated C-terminal tail of the Rad9 protein, which is recruited to ssDNA gaps as part of a checkpoint clamp complex.

Most checkpoint proteins and activities are conserved in all eukaryotic cells. In *Saccharomyces cerevisiae*, the orthologs of ATR and ATRIP are Mec1 and Ddc2, which form a stable complex required for checkpoint signaling in response to replication stress and DNA damage. Ddc2-deficient yeast exhibit hypersensitivity to DNA damage, and Ddc2 is required for phosphorylation of Mec1 substrates in response to DNA damage (Paciotti et al. 2000; Rouse and Jackson 2000). The homolog of TopBP1 in *S. cerevisiae* is thought to be Dpb11. However, Dpb11 lacks sequence homology with the AAD of TopBP1, and it is unclear whether it is a direct activator of Mec1. In at least some circumstances, the ortholog of hRad9 (Ddc1) is capable of activating Mec1–Ddc2 complexes in vitro (Majka et al. 2006).

To gain insight into how TopBP1 activates ATR–ATRIP complexes, we examined the interaction between these proteins. We identified a conserved region of ATRIP that is necessary for the interaction of ATR–ATRIP with TopBP1 and TopBP1-dependent ATR activation. We also defined a regulatory domain within ATR that mediates TopBP1-dependent ATR kinase activation. The ATR regulatory domain maps to the region between the kinase and FATC domains. This region is important for regulation of multiple PIK kinases including mTOR, ATM, and DNA-PKcs suggesting that divergent sequences within this region provide unique regulatory opportunities for each of these kinases.

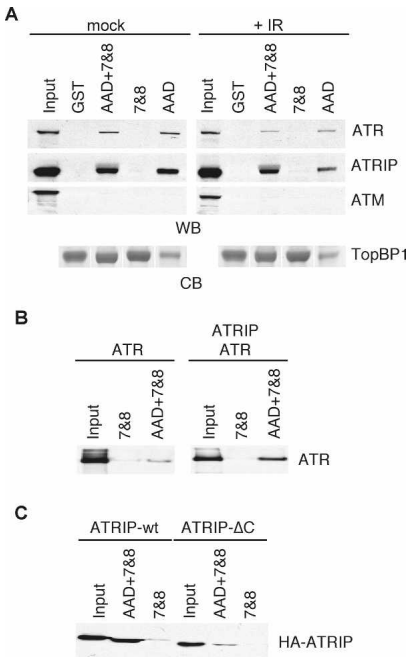

Figure 1. ATRIP promotes the association of ATR and TopBP1. (*A*) Nuclear extracts from 293T cells treated with 8 Gy of IR or mock-treated were incubated with equal amounts of recombinant GST-tagged fragments of TopBP1 fragments ([AAD] amino acids 978–1286 [Kumagai et al. 2006]; [7&8] BRCT repeats 7 and 8, amino acids 1182–1522; [AAD + 7&8] amino acids 978–1522) bound to glutathione beads. Proteins bound to the beads were eluted, separated by SDS-PAGE, and immunoblotted with antibodies to ATR, ATRIP, or ATM (WB). A duplicate gel was stained with Coomassie blue to verify equal amounts of GST-tagged TopBP1 proteins (CB). (*B*) Nuclear extracts from 293T cells transfected with a vector encoding ATR or vectors encoding ATR and ATRIP were incubated with recombinant fragments of TopBP1 bound to glutathione beads. Bound proteins were eluted, separated by SDS-PAGE, and immunoblotted with an antibody to ATR. (*C*) Nuclear extracts from U2OS cells stably expressing HA-tagged wild-type (wt) ATRIP or HA-tagged ATRIP lacking the C-terminal 32 amino acids (ΔC) were incubated with recombinant GST-tagged fragments of TopBP1 bound to glutathione beads. Proteins bound to the beads were eluted, separated by SDS-PAGE, and blotted with an anti-HA antibody. Input in all experiments is 5% of the extract added to the binding reaction.

Results

ATRIP promotes the association of ATR and TopBP1

To understand how TopBP1 activates the ATR–ATRIP complex, we sought to examine the interaction between these proteins. Nuclear extracts from mammalian cells were incubated with recombinant GST-tagged TopBP1 fragments. Endogenous ATR and ATRIP associated with TopBP1 fragments containing the AAD, but not with a C-terminal fragment of TopBP1 lacking the AAD or with GST alone (Fig. 1A). The amount of ATR and ATRIP associated with TopBP1 was not reproducibly affected by treating cells with ionizing radiation. An interaction between xATM and recombinant xTopBP1 has been demonstrated in *Xenopus* egg extracts and is potentiated by DNA templates that mimic DNA damage (Yoo et al. 2007). However, we were unable to detect an association between human ATM and TopBP1 even in the presence of DNA damage (Fig. 1A). Thus, the observed association with the TopBP1 AAD is specific to the ATR kinase.

Since TopBP1 stimulation of ATR kinase activity requires ATRIP (Kumagai et al. 2006; Ball et al. 2007), TopBP1 may interact with ATRIP, or ATRIP may stabilize an interaction between TopBP1 and ATR. Indeed, when ATR was transiently overexpressed in mammalian cells, the association of ATR with TopBP1 was significantly increased by simultaneous overexpression of ATRIP (Fig. 1B). This observation is consistent with studies performed in *Xenopus* egg extracts that indicate

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that xTopBP1 depends on the presence of xATRIP to associate with xATR–xATRIP (Kumagai et al. 2006). To test if ATRIP can associate with TopBP1 independently of ATR, nuclear extracts from U2OS cells expressing wild-type ATRIP or mutant ATRIP lacking the C-terminal ATR-interacting domain (Ball et al. 2005; Falck et al. 2005) were incubated with recombinant TopBP1 fragments. The binding of this ATRIPΔC mutant to TopBP1 is severely reduced compared with wild-type ATRIP (Fig. 1C). Taken together, these data suggest that the association of the ATR–ATRIP complex with TopBP1 might involve binding surfaces on both ATR and ATRIP.

Identification of a TopBP1-interacting region of ATRIP

To search for TopBP1-binding surfaces on the ATRIP and ATR proteins, we used a yeast two-hybrid approach. Given that the TopBP1 AAD is sufficient to associate with the ATR–ATRIP complex, the TopBP1 AAD fused to the GAL4 DNA-binding domain was used as a bait to screen a library containing thousands of random fragments of ATRIP fused to the activation domain of GAL4. Sequencing of ATRIP fragments selected in the screening procedure revealed a minimal interacting region of ATRIP consisting of amino acids 203–348 (Fig. 2A). This region is adjacent to the predicted ATRIP coiled-coil domain (amino acids 108–217), which mediates homo-oligomerization (Ball and Cortez 2005; Itakura et al. 2005). Sequence conservation of this region among ATRIP homologs is low except for a section of ~30 amino acids starting at amino acid 308 (Supplemental Fig. S1A). Deletion of amino acids 301–338 of ATRIP eliminated activation of ATR by TopBP1 in vitro, but also caused reduced binding to ATR (data not shown). An amino acid substitution mutation in this region of ATRIP (LLSS332AAAA) nearly abolished the association of ATRIP with TopBP1 (Fig. 2B), yet preserved the ability of ATRIP to bind ATR (Fig. 2C) and localize to damage-induced foci (data not shown). Furthermore, the association of ATR with TopBP1 was also significantly reduced in the presence of this ATRIP-*top* mutant compared with wild-type ATRIP (Fig. 2B), which supports the idea that ATR association with TopBP1 is dependent on ATRIP.

Since the ATR–ATRIP complex containing the ATRIP-*top* mutant had decreased association with TopBP1, we expected that it would have a decreased ability to be activated by TopBP1 as well. To test this hypothesis, wild-type ATR–ATRIP and mutant ATR–ATRIP-*top* complexes were immunopurified from mammalian nuclear extracts. Addition of the TopBP1 AAD to wild-type ATR–ATRIP complexes caused a robust stimulation of ATR kinase activity toward a substrate of ATR, MCM2 (Cortez et al. 2004; Yoo et al. 2004). The ATRIP-*top* mutant severely attenuated TopBP1-dependent stimulation of ATR (Fig. 2C). Thus, a previously uncharacterized domain of ATRIP is important for an interaction between TopBP1 and the ATR–ATRIP complex and for ATR activation by TopBP1.

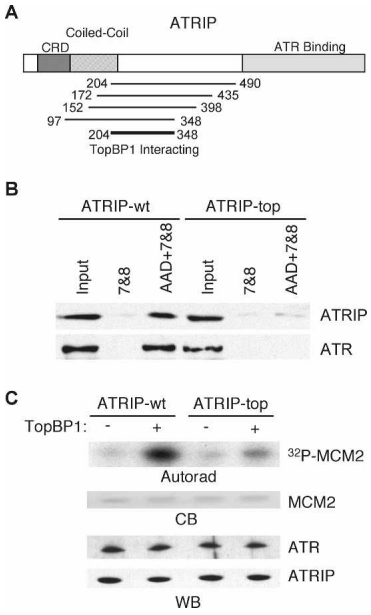

Figure 2. Identification of an ATRIP region necessary for TopBP1 association. (*A*) Schematic diagram showing the fragments of ATRIP that interacted with TopBP1 in a yeast two-hybrid assay (black lines). (CRD) Checkpoint Recruitment Domain (Ball et al. 2007). (*B*) Nuclear extracts from 293T cells transfected with vectors encoding ATR and wild-type ATRIP (wt) or ATR and ATRIP-*top* were incubated with recombinant GST-tagged fragments of TopBP1 bound to glutathione beads. Proteins bound to the beads were eluted, separated by SDS-PAGE, and blotted with antibodies to ATR or ATRIP. (*C*) Wild-type ATR–ATRIP (wt) or ATR–ATRIP-*top* complexes were isolated from transfected 293T cells and incubated with recombinant TopBP1 AAD, MCM2 substrate, and γ-^{32}PATP. Kinase reactions were separated by SDS-PAGE, stained with Coomassie blue (CB), and exposed to film (autorad). A duplicate gel was blotted and probed with anti-ATRIP and anti-ATR antibodies (WB).

The interaction between TopBP1 and ATRIP is essential for checkpoint responses to replication stress

A critical function of the ATR-dependent checkpoint signaling pathway in preserving genomic stability is to promote cell cycle recovery after replication fork arrest (Casper et al. 2002; Zachos et al. 2003). Since the ATRIP-*top* mutation selectively impairs TopBP1 association with and activation of ATR–ATRIP without impairing complex stability or localization to sites of DNA damage, this separation-of-function mutant provides a method to specifically assess the functional importance of TopBP1-dependent ATR activation. This is especially important given the possibility that RNAi depletion of TopBP1 likely interferes with many cellular processes in addition to checkpoints, such as replication control, making it difficult to unambiguously interpret phenotypic effects. To assess whether TopBP1 binding to ATRIP and activation of ATR is necessary for cells to display a normal checkpoint response to stalled replication forks, we created U2OS cell lines stably expressing siRNA-resistant wild-type ATRIP, the ATRIP-*top* mu-

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tant, or empty vector as a control. Three days after transfection with siRNA targeting endogenous ATRIP, the levels of wild-type ATRIP and ATRIP-*top* were comparable (Fig. 3B). The cell cycle profile of asynchronously dividing cells expressing ATRIP-*top* is similar to cells expressing wild-type ATRIP (Fig. 3A), indicating that ATRIP-*top* did not affect normal cell cycle progression. These cell lines were treated with hydroxyurea (HU), which causes replication forks to stall and arrests cells in early S phase. After 24 h, the HU was removed, and cells were released into media containing nocodazole to prevent cell division. The ability of the *ATRIP-top* mutant to support recovery and completion of DNA replication was compared with wild-type ATRIP. Sixteen hours after release from HU, most of the cells expressing wild-type ATRIP had completed S phase (Fig. 3A). Yet, the majority of ATRIP-depleted cells expressing the ATRIP-*top* mutant or lacking any exogenous ATRIP were unable to resume DNA replication and complete S phase. Consistent with the reduced ability of ATRIP-*top* cells to recover from replication stress, these cells also displayed significantly reduced viability compared with wild-type ATRIP-expressing cells after treatment with HU (Fig. 3C). The ATRIP-*top* cells also exhibited a defect in the G2/M checkpoint after treatment with ionizing radiation (Fig. 3D). Therefore, the ability of ATR–ATRIP complexes to promote recovery from stalled replication forks and cell cycle arrest after DNA damage is dependent on an interaction between TopBP1 and ATRIP that leads to ATR kinase activation.

Conservation of the TopBP1-dependent ATRIP regulatory domain in S. cerevisiae

It is unclear whether the mechanism of activation of Mec1–Ddc2 complexes in *S. cerevisiae* is the same as ATR–ATRIP since no yeast protein has obvious sequence similarity to the AAD of TopBP1. To address whether the mechanism of regulation for Mec1–Ddc2 complexes is likely to be similar to ATR–ATRIP, we introduced a mutation into Ddc2 that is analogous to the ATRIP-*top* mutant. Despite little sequence similarity, Ddc2 and ATRIP have a common functional domain architecture: an N-terminal checkpoint recruitment domain, a predicted coiled-coil domain, and a C-terminal domain essential for interaction with Mec1 or ATR, respectively (Wakayama et al. 2001; Ball et al. 2005, 2007; Falck et al. 2005). To generate a *ddc2-top* mutation that corresponds to the *ATRIP-top* mutation, we used position and secondary structure predictions as a guide (Fig. 4A). Specifically, we mutated leucine residues that were approximately the same number (115) of amino acids C-terminal from the end of the predicted coiled-coil domain and within the third predicted α-helix (*-top*: LLLR257AAAA). For comparison, we made two additional *ddc2* alleles with mutations in the fourth predicted α-helix after the coiled-coil domain (*-A1*: LLED274AAAA and *-A2*: LIKE281AAAA). Mutant or wild-type Ddc2 were expressed on a low-copy plasmid under the endogenous *DDC2* promoter in a *ddc2*Δ strain (Paciotti et al. 2000; Rouse and Jackson 2002). The *top*, *A1*, and *A2* mutants were expressed at comparable levels as wild-type Ddc2 and are able to interact with Mec1 equivalently to wild-type Ddc2 (Fig. 4B; data not shown). The *top* mutation but not the *A1* or *A2* mutation caused marked sensitivity to HU and the alkalyating agent methyl methanesulfonate (MMS) (Fig. 4C). Furthermore, the sensitivity to DNA damage of the *ddc2-top* strain correlated with reduced phosphorylation of the Mec1 substrate Rad53 following a challenge with either HU or MMS, suggesting that disruption of this Ddc2 domain causes a defect in Mec1 activation (Fig. 4D). These data suggest that although the protein equivalent to the

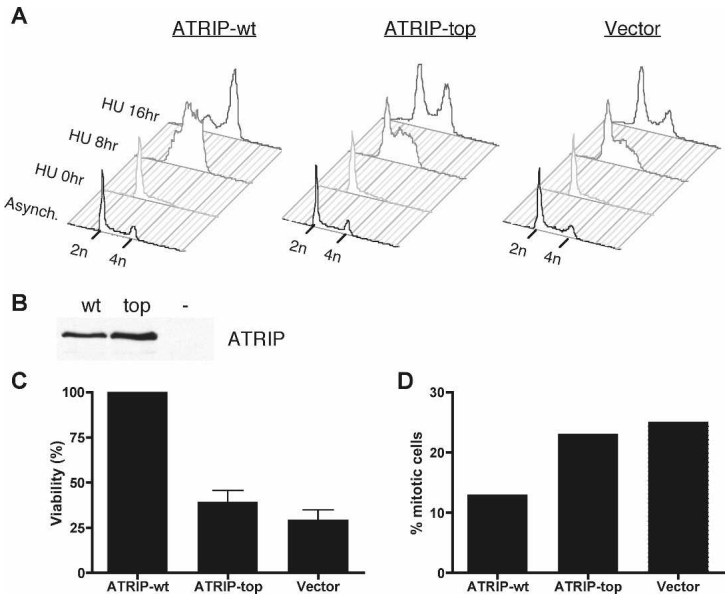

Figure 3. ATRIP association with TopBP1 is essential for cellular recovery from replication stress. (*A–C*) U2OS cells stably expressing siRNA-resistant wild-type ATRIP (wt), ATRIP-*top*, or an empty vector (vector) were transfected with siRNA targeting ATRIP to deplete endogenous ATRIP. Three days later, cells were exposed to 1 mM HU for 24 h. (*A*) Cells were collected immediately (0 hr) or rinsed and released into media containing 1 µg/mL nocodazole for either 8 h (8 hr) or 16 h (16 hr). Cells were fixed and stained with propidium iodine and processed for FACS analysis. (Asynch) Asynchronous cells that were not exposed to HU. (*B*) Immunoblot showing ATRIP levels from U2OS cells stably expressing wild-type ATRIP (wt), ATRIP-*top* (*top*), or empty vector (vt). (*C*) Twenty-four hours after release from HU, cellular viability was measured using a colorimetric assay. Viability was normalized to cells expressing exogenous wild-type ATRIP. Error bars indicate standard error, $n = 6$. (*D*) Three days after siRNA transfection, cells were treated with 4 Gy of IR, and 1 µg/mL nocodazole was added to the media. Sixteen hours later, the percentage of mitotic cells were determined by propidium iodine and antiphosphohistone H3 staining followed by flow cytometry.

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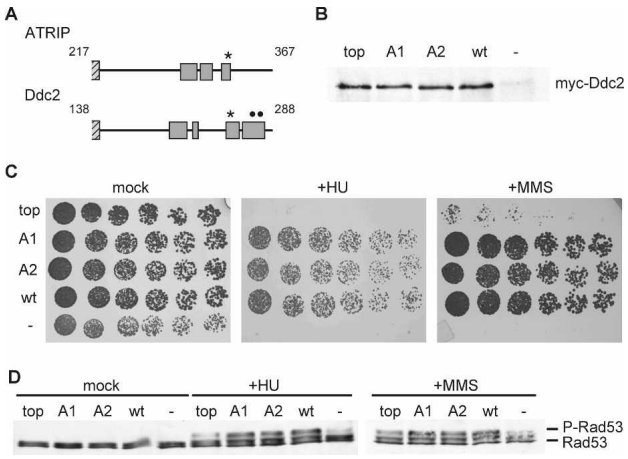

Figure 4. An *S. cerevisiae ddc2-top* mutant is defective in checkpoint signaling. (*A*) Secondary structure prediction of the TopBP1-interacting region of ATRIP and the equivalent region of *S. cerevisiae* Ddc2. Hashed boxes denote the C-terminal ends of the coiled-coil domains. Solid boxes indicate predicted α-helices. The asterisk denotes the location of the *ATRIP-top* mutation or the *ddc2-top* (LLLR257AAAA) mutation. The adjacent dots in Ddc2 denote the location of the *A1* (LLED274AAAA) and *A2* (LIKE281AAAA) mutations. (*B*) Immunoblot showing Ddc2 levels in yeast strains expressing Ddc2 mutants, wild-type Ddc2, or empty vector. (*C*) Serial dilutions of the indicated yeast strains grown on YPD with no drug (mock), 150 mM HU (+HU), or 0.008% MMS (+MMS). (*D*) Exponentially growing yeast were treated with no drug (mock), 150 mM HU (+HU), or 0.015% MMS (+MMS) for 90 min. Extracts were prepared, separated by SDS-PAGE, and immunoblotted with an antibody against Rad53. The *top* bands are phosphorylated forms of Rad53.

TopBP1 ATR activator in budding yeast is unclear, a common mechanism of regulation dependent on the ATRIP (or Ddc2) protein is likely to exist for vertebrate and yeast ATR kinases.

Regulation of ATR activation via a PIKK regulatory domain

Since the ATR-binding-defective ATRIPΔC protein does not bind as well to TopBP1 as wild-type ATRIP (Fig. 1), we hypothesized that regions of ATR contribute to the interaction between TopBP1 and the ATR–ATRIP complex. ATR has similar domain architecture to other members of the PIKK protein kinase family. It contains dozens of N-terminal HEAT repeats and a kinase domain flanked by FAT and FATC domains (Fig. 5A). The N-terminal heat repeats provide a binding surface for ATRIP (Ball et al. 2005; Chen et al. 2007). Aside from this, little is known about the structure of ATR. We again used the TopBP1 AAD as bait in a yeast two-hybrid approach to screen a library of thousands of ATR fragments. All of the interacting fragments recovered contained amino acids 2483–2597 of ATR (Fig. 5A). These residues span the C-terminal end of the kinase domain and an uncharacterized region of ATR between the kinase and FATC domains, which we named the PIKK Regulatory Domain (PRD) for reasons discussed below.

Although the kinase and FATC domains share a high degree of sequence similarity among all PIKK family members, the sequence of the PRD is highly divergent in these paralogs (Supplemental Fig. S1B). However, the PRD has a high degree of sequence identity within orthologous ATR proteins from different organisms. Deletion of the entire ATR PRD abolished all kinase activity (data not shown), probably as a result of disrupting the folding of the adjacent kinase domain. Scanning mutagenesis of the ATR PRD identified two mutations (K2589E and HVL2591AAA) that largely eliminated TopBP1-activation of ATR kinase activity without changing the basal activity of the kinase in the absence of TopBP1 (Fig. 5B; Supplemental Fig. S2). Several other mutations in this region including a small deletion Δ2569–2576 and a charge reversal of a lysine one amino acid separated from K2589 (K2587E) had no significant effect on either the basal or TopBP1-activated ATR kinase activity (Fig. 5B,C; Supplemental Fig. S2). None of these mutations had any effect on the ability of ATR to bind ATRIP. Mutation of K2589 to alanine did not impair ATR activation (Supplemental Fig. S2A), indicating that a post-translation modification at this site is not necessary for ATR activation by TopBP1.

Since ATR K2589E has equivalent basal kinase activity toward itself, ATRIP, and a substrate as wild-type ATR (Fig. 5D), the mutation does not alter the catalytic activity of ATR in the unactivated state but specifically affects the formation of the active ATR protein. Additionally, this mutation does not affect the ability of ATR to form homo-oligomeric complexes (Supplemental Fig. S3).

Next, we tested the ability of ATR–ATRIP complexes containing ATR K2589E to associate with TopBP1. The ATR K2589E mutant modestly decreased the association with TopBP1 (approximately twofold) in pull-down assays compared with wild-type ATR–ATRIP complexes (Fig. 5E). The remaining association is likely mediated by the ATRIP–TopBP1-binding interface.

Regulation of PIK kinases via the PIK regulatory and FATC domains

A recent study suggested that the region of ATM equivalent to the ATR PRD is targeted for acetylation by the Tip60 histone acetylase (Sun et al. 2007). The DNA-damaged inducible acetylation of a specific lysine in the ATM PRD is necessary for the increased kinase activity of ATM after DNA damage. To test whether acetylation of the ATR PRD might regulate its activation by TopBP1, we made lysine-to-arginine mutations in the PRD. None of these mutations, even when combined into a single ATR protein, had any effect on TopBP1-mediated activation of ATR in vitro (Supplemental Fig. S2B). Also, siRNA depletion of Tip60 did not affect the ability of cells to recover from HU (data not shown). Hence, the PRD of ATR is likely not regulated through acetylation in the same manner as the PRD of ATM.

To determine if the PRD is necessary for the regulation of other PIKK family members besides ATR and

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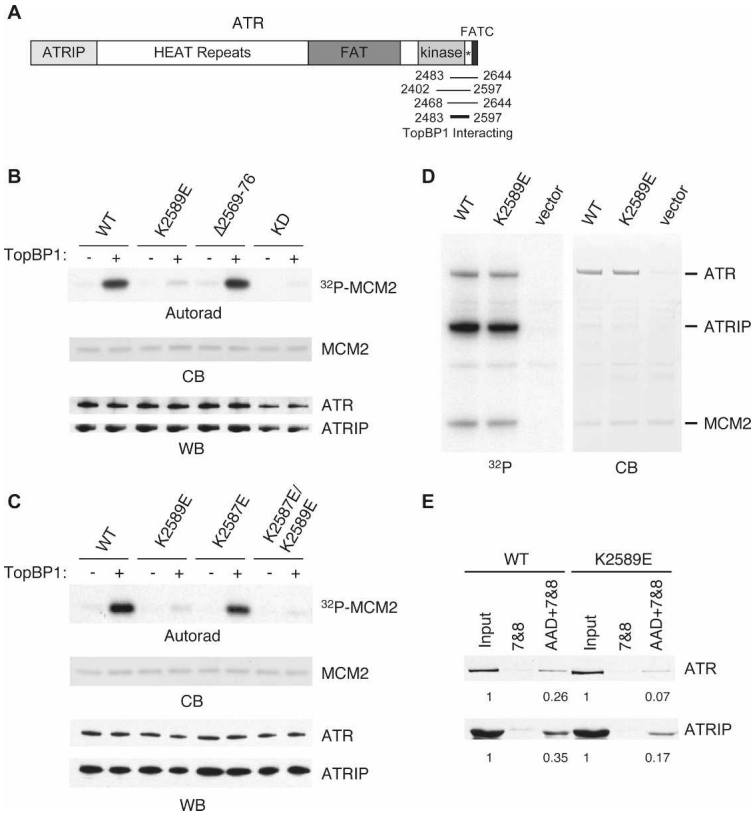

Figure 5. An ATR regulatory region between the kinase and FATC domains is critical for TopBP1-dependent activation of ATR in vitro. (*A*) Schematic diagram showing the domains of ATR and the ATR fragments that interact with TopBP1 in a yeast two-hybrid assay (black lines). (*B*) Wild-type ATR (WT), ATR K2589E, ATR Δ2569–2576, or ATR kinase dead (KD) proteins complexed with wild-type ATRIP were isolated from transfected 293T cells and incubated with MCM2 substrate, γ-^{32}P-ATP, and recombinant TopBP1 AAD where indicated. Kinase reactions were separated by SDS-PAGE, stained with Coomassie blue (CB), and exposed to film (autorad). A duplicate gel was immunoblotted with anti-ATRIP and anti-ATR antibodies (WB). (*C*) Kinase reactions to measure TopBP1-dependent activation of wild-type ATR (WT), ATR K2589E, ATR K2587E, or ATR K2587E/K2589E were performed as in *B*. (*D*) To measure basal kinase activity plasmids encoding ATRIP and Flag-tagged wild-type ATR (WT), ATR K2589E, or empty vector were expressed in 293T cells and immunoprecipitated with anti-Flag antibodies. Complexes were incubated with MCM2 substrate and γ-^{32}PATP. Kinase reactions were separated by SDS-PAGE, stained with Coomassie blue (CB), and exposed to film (autorad). (*E*) Nuclear extracts from 293T cells transfected with vectors encoding wild-type ATR (wt) and ATRIP or ATR K2589E and ATRIP were incubated with recombinant fragments of TopBP1 bound to glutathione beads. Bound proteins were eluted, separated by SDS-PAGE, and immunoblotted with ATR or ATRIP antibodies. Input equals 5% of the extract used in the binding reactions. Quantification of the immunoblot signal normalized to the input is shown.

ATM, we examined the PRD of DNA-PKcs. Activation of DNA-PKcs requires the Ku70/80 heterodimer and DNA ends (Smith and Jackson 1999). Electron microscopy structural studies of the DNA-PK complex demonstrated an interaction between the Ku70/80 heterodimer and a region immediately C-terminal to the kinase domain, suggesting that Ku70/80 binding could be mediated in part by the DNA-PKcs PRD (Spagnolo et al. 2006). Therefore, we tested if the PRD is necessary for the activation of DNA-PKcs. In response to dsDNA breaks, DNA-PKcs is autophosphorylated at Ser 2056, an event that is required for nonhomologous end-joining-mediated DNA double-strand break repair (Chen et al. 2005). Wild-type DNA-PKcs and DNA-PKcs PRD mutants (M1: K4043E/K4048E/R4049E/K4050E, M2: D4062K/E4063K/E4069K, M3: K4075E/R4082E/R4085E/R4090E) were expressed in DNA-PKcs-defective cells. Cells were exposed to ionizing radiation and the phosphorylation status of Ser 2056 was assessed. Although wild-type DNA-PKcs and the M2 DNA-PKcs PRD mutant exhibited Ser 2056 phosphorylation, the M1 and M3 DNA-PKcs PRD mutants did not (Supplemental Fig. S4). Thus, specific residues within the PRD of DNA-PKcs are required for DNA-PKcs autophosphorylation, suggesting that the PRD is important for the regulation of DNA-PKcs as well.

Since the FATC domain amino acid sequence is highly conserved among PIK kinases and adjacent to the PRD, we tested if it is also important for TopBP1 to activate ATR. Deletion of part of or the entire FATC domain abolished even the basal kinase activity of ATR (Supplemental Fig. S5). Since the FATC domain of ATR can substitute for the FATC domain of ATM (Jiang et al. 2006), we performed the reciprocal experiment. Replacement of the FATC domain of ATR with that of ATM also resulted in a kinase-dead mutant, indicating that it cannot substitute (Supplemental Fig. S5). Thus, the FATC domain of ATR is essential for even basal ATR kinase activity.

ATR regulation through the PRD is essential for checkpoint signaling and cell viability

The ATR-PRD mutation provides a second separation of function mutant useful for examining the functional importance of TopBP1-mediated ATR activation. To determine if regulation of ATR by TopBP1 through the PRD is necessary for cellular responses to replication stress, we created ATR$^{flox/-}$ cell lines expressing a Tet-inducible form of either wild-type ATR or mutant K2589E ATR. The cell lines were treated with tetracycline to induce expression of the exogenous ATR prior to deletion of the floxed ATR allele with the Cre recombinase. Expression levels of the wild-type or K2589E ATR protein were similar and near the endogenous amount of ATR expression (Fig. 6A). Phosphorylation of the essential kinase

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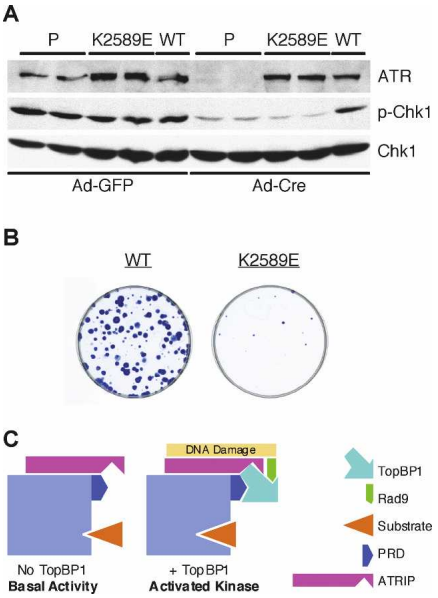

Figure 6. ATR K2589E does not support checkpoint signaling. (*A*) ATR$^{flox/-}$ cell lines were created that contained an inducible form of either ATR K2589E or ATR wild-type (WT). P1 and P2 denote ATR$^{flox/-}$ parental cell lines lacking any exogenous ATR. The cells were induced to express exogenous ATR with tetracycline and were treated with adenovirus encoding the Cre recombinase (Ad-Cre) to delete the endogenous ATR or adenovirus expressing GFP (Ad-GFP) as a control. Four days after infection, cells were treated with 1 mM HU for 6 h. Cell lysates were separated by SDS-PAGE and blotted with the indicated antibodies to ATR, Chk1, or phosphorylated Chk1. (*B*) Equal numbers of Cre-infected ATR$^{flox/-}$ cells expressing wild-type ATR or ATR K2589E were plated. Seventeen days after plating, the surviving colonies were stained with methylene blue. (*C*) Schematic model for ATR activation. In the absence of TopBP1, ATR exhibits a low basal kinase activity. In response to genotoxic stress, ATRIP recruits ATR to sites of DNA damage. Loading of the Rad9–Hus1–Rad1 complex allows Rad9 to recruit TopBP1. TopBP1 makes contact with both ATRIP and the ATR PRD. The interaction between TopBP1 and ATR–ATRIP greatly stimulates ATR kinase activity, perhaps due to a conformational change in the ATR kinase domain that facilitates the ability of ATR to interact with its substrates.

Chk1, an ATR substrate (Liu et al. 2000), was used to assess ATR signaling in cells. Robust Chk1 phosphorylation following HU treatment is observed in all the cell lines prior to deletion of the endogenous ATR allele (Fig. 6A). However, the K2589E ATR protein failed to support Chk1 phosphorylation after HU treatment, yielding phospho-Chk1 levels comparable with cells not expressing any exogenous ATR. In contrast, wild-type, exogenous ATR could support Chk1 phosphorylation in cells lacking endogenous ATR (Fig. 6A, last lane). Two K2589E ATR-expressing clonal cell lines yielded identical results. Thus, ATR K2589E is defective in checkpoint signaling after replication stress.

ATR is essential for the viability of proliferating cells, but its essential function is not clear (Brown and Baltimore 2000; Cortez et al. 2001). Given that the K2589E mutant ATR could not support checkpoint signaling, we wanted to assay whether this mutant would be capable of supporting cellular viability even in the absence of exogenously added genotoxic agents. The ATR$^{flox/-}$ cells lines stably expressing wild-type or mutant K2589E ATR were treated with adenovirus expressing Cre recombinase and plated at low density. After 17 d, surviving cell colonies were stained. The colony formation assay revealed a dramatic difference in the number of colonies between the two cell lines (Fig. 6B). Only a few colonies grew from the mutant cell line. PCR genotyping of these colonies from a duplicate sample indicated that all the surviving colonies expressing the K2589E ATR had not undergone Cre-mediated recombination to delete the endogenous ATR allele; whereas, all of the colonies expressing wild-type ATR underwent Cre-mediated recombination and lacked the endogenous ATR allele (Supplemental Fig. S6). Thus, although we could obtain an ATR$^{-/-}$ cell line rescued by a wild-type ATR cDNA, we were not able to obtain an ATR$^{-/-}$ cell line expressing only the K2589E ATR mutant. This suggests that TopBP1-mediated activation of ATR is essential for the viability of, at least, this human cell type in culture.

Discussion

The PIK kinases regulate many cellular responses including nutrient sensing and the DNA damage response. Therefore, their activities impact many human diseases. Unfortunately, their large size and atypical kinase domains have made understanding their activation mechanisms difficult. In this study, we define how the TopBP1 activator protein binds to the ATR–ATRIP complex and identify critical regulatory regions within both the ATRIP and ATR proteins. Importantly, we provide evidence that the ATRIP regulatory region is conserved functionally in the yeast ATRIP protein Ddc2, and the ATR regulatory domain is a common site for regulation of most, if not all, of the PIK kinases.

Our data support the ATR activation model shown in Figure 6C. In the absence of DNA damage or replication stress, ATR has basal kinase activity. Following a challenge to the genome that exposes ssDNA gaps, ATRIP and the 9–1–1 checkpoint clamp are recruited independently (Melo et al. 2001; Zou et al. 2002). ATRIP brings ATR and Rad9 brings TopBP1. The assembly and concentration of these components at sites of DNA damage facilitates an interaction between TopBP1 and interacting surfaces on both ATRIP and ATR. This interaction then promotes ATR activation.

Additional regulatory steps are clearly important. For example, the ATR–ATRIP complex, 9–1–1 checkpoint clamp, and TopBP1 are phosphorylated, suggesting that post-translational modifications may fine-tune ATR activation (Roos-Mattjus et al. 2003; Myers et al. 2007; Venere et al. 2007; Yoo et al. 2007). Furthermore, specific ATR substrates (such as Chk1) require additional protein cofactors (such as Claspin) to be efficiently phosphorylated (Kumagai and Dunphy 2000; Liu et al. 2006). Exactly how TopBP1 binding increases ATR kinase activity

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will require a structural description of these proteins. One possibility is that it alters the conformation of the ATR kinase domain such that substrates can access ATR more easily. Consistent with this interpretation, we found that TopBP1 binding to ATR decreases the apparent K_m of ATR for substrates (D.A. Mordes, unpubl.).

ATRIP provides several functions to regulate ATR activation

ATR function is dependent on its binding partner ATRIP. ATRIP provides at least four activities that promote ATR signaling. First, it stabilizes ATR (Cortez et al. 2001). Second, it promotes the localization of ATR to sites of DNA damage or replication stress (Zou and Elledge 2003; Ball et al. 2005, 2007). Third, ATRIP post-translational modifications regulate ATR signaling (Myers et al. 2007; Venere et al. 2007). Finally, as demonstrated in this report, ATRIP binds directly to TopBP1, and this binding is essential for ATR activation.

Mutations of the TopBP1-interacting region of ATRIP impair the ability of cells to recover from replication stress and arrest the cell cycle after DNA damage. Mutations of the same region in *S. cerevisiae* ATRIP (Ddc2) cause sensitivity to replication stress and impair activation of Mec1 in response to DNA damage. This suggests that the mechanism of activation for ATR may be conserved throughout evolution. Although Mec1 and Ddc2 are clear orthologs of ATR and ATRIP, respectively, no obvious *S. cerevisiae* ortholog exists for TopBP1. Two checkpoint proteins, Dpb11 and Ddc1, are candidates for protein activators of Mec1, but neither share sequence identity to the TopBP1 AAD. Like TopBP1, Dpb11 is a BRCT-repeat-containing protein that has essential roles in replication and checkpoint signaling. Yet, Dpb11 has not been reported to activate Mec1 directly. In vertebrates, TopBP1 is recruited to gapped ssDNA regions through an interaction with the Rad9 subunit of the checkpoint clamp (Delacroix et al. 2007; Lee et al. 2007). Ddc1 is the yeast ortholog of Rad9 and also binds to Dpb11 (Wang and Elledge 2002). The C-terminal tail of Ddc1 stimulates the kinase activity of the Mec1–Ddc2 complex in vitro under low-salt conditions (Majka et al. 2006). However, overexpression of this portion of Ddc1 is not sufficient to cause activation of Mec1. In contrast, overexpression of the TopBP1 AAD is sufficient to cause pan-nuclear activation of ATR in mammalian cells (Kumagai et al. 2006; Ball et al. 2007). It is possible that both proteins may act as activators of Mec1 in response to different types of DNA damage or they may form an activator complex. In any case, our results predict that a Mec1 activator is likely to bind to a surface on Ddc2 encoded just C-terminal of the coiled-coil domain containing amino acids 257–260.

PIK kinase regulation

PIKKs are key regulators of many critical signaling pathways, including cell growth, cell cycle checkpoints, and DNA damage. Despite their diverse functions, emerging data suggest many similarities among them. PIKKs associate with an interacting partner (ATRIP for ATR, NBS1 for ATM, Ku70/80 for DNA-PKcs, and Rictor and Raptor for mTOR) that is critical for their function and, in the case of ATRIP, NBS1, and Ku80, their localization to sites of DNA damage (Cortez et al. 2001; Hara et al. 2002; Sarbassov et al. 2004; Falck et al. 2005). PIKKs also require an interaction with another regulatory protein or protein complex for their activation. GβL (mLST8) binds to the kinase domain of mTOR and stimulates its catalytic activity (Kim et al. 2003; Wullschleger et al. 2005). Rheb also activates mTOR–Raptor complexes (Long et al. 2005). The Mre11/Rad50 complex makes multiple contacts with ATM and is sufficient to stimulate its kinase activity especially in the presence of DNA (Lee and Paull 2004). The Ku70/Ku80 complex stimulates DNA-PKcs in the presence of DNA ends (Smith and Jackson 1999). Finally, TopBP1 stimulates the kinase activity of ATR in an ATRIP-dependent manner (Kumagai et al. 2006). There are apparently even common regulators of most, if not all, the PIK kinases. Recently, Tel2 has been demonstrated to interact with all PIKKs and regulate their stability (Takai et al. 2007).

Given the similarity of sequence and structure of the PIK kinases, it might be expected that the mechanisms controlling their activation would be similar (although responsive to different inputs). Indeed, the FATC domain is highly conserved in sequence among PIKKs and is functionally interchangeable in some instances (Jiang et al. 2006). The FATC domain is required for the kinase activity of ATM, DNA-PKcs, mTOR, and SMG-1 (Banin et al. 1998; Priestley et al. 1998; Takahashi et al. 2000; Morita et al. 2007). Our data indicate that the FATC domain of ATR is essential for its kinase activity as well. We noticed that mutation of the FATC domain of ATR caused a reduction in expression levels, and a similar observation has been made for ATM (Jiang et al. 2006). Since all kinase activity is lost when the FATC domain is mutated, we suspect that the FATC domain may be important for the stability of PIKKs or for proper folding of the kinase domain. One possibility is that the FATC domain makes critical contacts within the kinase or FAT domains that stabilize the kinase domain.

In contrast to FATC domain alterations, mutations in the PRD (the region between the kinase and FATC domains) of ATM, ATR (this study), mTOR, and SMG-1 do not abolish PIK kinase activity (Sekulic et al. 2000; Morita et al. 2007; Sun et al. 2007). However, these mutations do impair kinase regulation. This region does not exhibit sequence similarity among PIKKs, suggesting that it could be sensitive to different regulatory inputs for each of the kinases. The PRD domain of mTOR contains an Akt phosphorylation site that regulates its activity (Sekulic et al. 2000). The Tip60 histone acetyltransferase complex acetylates ATM within the ATM PRD, and this acetylation is important for the activation of ATM kinase activity after DNA damage (Sun et al. 2005, 2007). We now show that the ATR PRD is critical for TopBP1-dependent activation of ATR and the PRD of

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DNA-PKcs is necessary for its autophosphorylation. Despite the similarity of ATM and ATR, we have no evidence that the ATR PRD is acetylated. In fact, mutations of all the lysines in this region to arginines did not impair TopBP1-dependent activation.

We propose that the PRD has evolved to allow distinct regulation of the kinase activity of the PIKK family members. Differences in the PRD and the PIK-binding partner provide specificity for the types of cellular events that can activate each PIKK. It will be interesting to determine whether the ATM PRD or mTOR PRD is important for Mre11/Rad50-dependent or GβL/Rheb-dependent ATM or mTOR activation, respectively. Also, it will be important to examine the impact of ATM PRD acetylation on Mre11/Rad50-dependent regulation.

Defects in cellular responses to replication stress in cells lacking TopBP1-dependent ATR activation

Previous studies have demonstrated critical roles for ATR signaling and the TopBP1 protein for replication stress-induced checkpoints. Furthermore, the C-terminal half of TopBP1 was shown to be essential for checkpoint signaling in *Xenopus laevis* egg extracts (Hashimoto et al. 2006; Kumagai et al. 2006; Yan et al. 2006). However, the interpretation of genetic experiments on TopBP1 in human cells is problematic given that it forms complexes with many other proteins and regulates both DNA replication and checkpoints. The analysis of the separation-of-function mutants that we created in both ATR and ATRIP allows us to unambiguously define the cellular requirements for TopBP1-dependent ATR activation. Our analysis reveals that not only is TopBP1-dependent ATR activation essential for checkpoint responses to replication stress, but it is also required for the essential function of ATR in promoting cellular viability.

Conclusions

Our data suggest both similarities and differences among the regulation of the PIKK family of protein kinases. The ability of TopBP1 to uniquely stimulate ATR is explained, in part, by the requirement of an ATRIP surface for the binding of TopBP1 to the ATR–ATRIP complex. The PRD of ATR and the other PIKKs is a second important regulatory determinant. The exact mechanism by which the PRD functions in PIKK activation remains to be determined, but given its position between the kinase and FATC domains, it seems likely that it mediates a conformational change that may allow greater kinase activity.

Inhibition of ATR sensitizes cancer cells to multiple DNA-damaging agents (Wilsker and Bunz 2007). Thus far, specific inhibitors of ATM and DNA-PKcs kinases have been developed and are being studied as potential therapeutic agents (Lord et al. 2006); however, inhibitors of the ATR kinase have not been isolated. Targeting the interaction between ATRIP and TopBP1 or the ATR PRD may provide a novel means for developing an agent to disrupt ATR signaling.

Materials and methods

Cell lines

HEK 293T and U2OS cell lines were maintained in DMEM + 7.5% fetal bovine serum. The V3 CHO cell line was maintained in αMEM supplemented with 10% fetal bovine serum, 10 µg/mL ciprofloxacin (Mediatech), 50 U/mL penicillin, and 50 µg/mL streptomycin (Invitrogen). Construction of ATRIP stable cell lines and use of ATRIP siRNA were as described previously (Ball et al. 2005), except transfections of siRNAs at 10 nM were performed with HiPerFect (Qiagen). Plasmid transfections of HEK 293T cells were performed with Lipofectamine 2000 (Invitrogen). Plasmid transfections of V3 CHO cells were performed with TransIT-CHO (Mirus Bio). HCT116 $ATR^{flox/-}$ cells were maintained in McCoy's medium + 7.5% fetal bovine serum. Clonal ATR stable lines were made by transfecting HA-tagged ATR tetracycline-inducible vectors into $ATR^{flox/-}$-TetR cells, and selecting with 300 µg/mL Hygromycin B (Invitrogen) for single colonies. ATR expression was induced with 1 µg/mL tetracycline (Invitrogen). Deletion of the ATR gene and PCR genotype analysis for the ATR allele was performed as described previously (Cortez et al. 2001). For the colony formation assays, equal numbers of cells were plated onto 60 mM tissue culture dishes and incubated for 17 d in the presence of 300 µg/mL Hygromycin B and 1 µg/mL tetracycline. Media was changed every 3 d. Colonies were stained with methylene blue (Sigma).

Yeast

Ddc2 mutations were made in the pNML1 centromeric plasmid encoding myc-Ddc2 under the endogeonous Ddc2 promoter (Rouse and Jackson 2002) and were expressed in strain DMP2995/1B: *MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sml1*Δ::*KanMX4 ddc2*Δ::*KanMX4* (Paciotti et al. 2000). Yeast protein samples were prepared using TCA precipitation as described (Longhese et al. 1997).

DNA constructs

PCR site-directed mutagenesis was performed using the QuickChange method and PfuUltra DNA polymerase (Stratagene). All constructs generated using PCR were confirmed by sequencing. The DNA-PKcs expression vector was kindly provided by Dr. Kathryn Meek. Cloning details for any construct are available upon request.

Protein interactions

TopBP1-binding assay: Recombinant GST-tagged TopBP1 fragments were purified from *Escherichia coli* with glutathione Sepharose 4B beads (GE Healthcare). Nuclear extracts were prepared as described (Kumagai et al. 2006) and incubated with the indicated GST-tagged proteins bound to glutathione Sepharose beads overnight at 4°C. Beads were washed three times in low-salt buffer (20 mM HEPES-KOH at pH 7.9, 175 mM NaCl, 20% glycerol, 0.05% Tween 20), and proteins were eluted and separated by SDS-PAGE prior to immunoblotting.

Two-hybrid experiments were performed using the TopBP1 AAD (amino acids 978–1286) cloned into pDAB1 containing the DNA-binding domain of Gal4. This bait was used to screen the

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pACT-ATRIP and pACT-ATR cDNA fragment libraries that we described previously (Ball et al. 2005) using the PJ694A yeast strain (James et al. 1996).

Kinase assays were performed largely as described previously (Cortez et al. 2001; Ball et al. 2007). Assays measuring the basal ATR activity were performed by immunoprecipitating Flag-ATR protein using anti-Flag M2 agarose beads (Sigma) from TGN buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 1% Tween 20, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 1 mM NaF, 50 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) cell lysates. Immunoprecipitates were washed three times in TGN buffer, once in TGN + 500 mM LiCl, and twice in kinase buffer (10 mM HEPES at pH 7.5, 50 mM NaCl, 50 mM β-glycerol phosphate, 10 mM $MgCl_2$, 1 mM diothiothreitol) prior to performing kinase reactions. Assays measuring TopBP1 stimulation of ATR activity were performed by immunoprecipitating Flag-ATR/HA-ATRIP complexes using anti-HA agarose beads (Sigma). Immunoprecipitates were washed three times in TGN buffer, once in TGN + 500 mM LiCl, and twice in kinase buffer prior to performing kinase reactions. GST-TopBP1 AAD was added to the reactions prior to adding ATP and substrate. All reactions were stopped within the linear range of the assay and analyzed by SDS-PAGE and autoradiography.

For experiments to assess ATR oligomerization, 293T cells transiently expressing the indicated plasmid were lysed in CHAPS lysis buffer as described (Ball and Cortez 2005).

Antibodies

The ATRIP-N antibody has been described previously (Cortez et al. 2001). The following antibodies were purchased: ATM (Novus), ATR and Chk1 (Santa Cruz Biotechnologies), Chk1 P317 (Cell Signaling), DNA-PKcs (Serotec), DNA-PKcs pS2056 (Abcam), HA.11 and Myc9E10 (Covance), and Flag M2 (Sigma). The Rad53 antibody was a gift from Stephen Elledge.

Bioinformatics

Protein sequence alignments were performed using ClustalW, Boxshade, and Coils (http://www.ch.embnet.org). Protein secondary structure predictions were generated by PsiPred (http://bioinf.cs.ucl.ac.uk/psipred).

HU recovery, viability, and checkpoint assays

Three days after siRNA transfection, cells were incubated in media with or without HU for 24 h. Cells were released into fresh media for 24 h, and cell viability was assessed using the WST-1 reagent assay (Roche), which is a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases. The percentage viability was calculated as the ratio of the 450 nM absorbance of the HU-treated cells to the untreated cells. The HU recovery assays were performed using the same protocol with the following differences: Nocodazole was added to the media when the HU was removed, and cells were harvested at 0, 8, or 16 h after release. Harvested cells were fixed in ethanol, stained with propidium iodide, and analyzed on a BD Biosciences FACSCalibur. A stock solution of HU (Sigma) was prepared in water at 1 M and stored frozen. Nocodazole (Acros) was dissolved in dimethylsulfoxide at 10 mg/mL. For the G2/M checkpoint assay, harvested cells were fixed in ethanol, permeabilized, stained with an antibody against H3 phospho-S10 antibody and an anti-FITC secondary antibody, propidium iodide, and then analyzed by FACS. Cells with 4n DNA content and H3 phospho-S10-positive were counted as mitotic cells.

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Dpb11 activates the Mec1-Ddc2 complex

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Abbreviations: ATR, ATM- and Rad3-related; BRCT, BRCA1 C-terminal; HU, hydroxyurea

Abstract

The *S. cerevisiae* Mec1-Ddc2 checkpoint kinase complex (the ortholog to human ATR-ATRIP) is an essential regulator of genomic integrity. The *S. cerevisiae* BRCT repeat protein Dpb11 functions in the initiation of both DNA replication and cell cycle checkpoints. Here, we report a genetic and physical interaction between Dpb11 and Mec1-Ddc2. A C-terminal domain of Dpb11 is sufficient to associate with Mec1-Ddc2 and strongly stimulates the kinase activity of Mec1 in a Ddc2-dependent manner. Furthermore, Mec1 phosphorylates Dpb11 and thereby amplifies the stimulating effect of Dpb11 on Mec1-Ddc2 kinase activity. Thus, Dpb11 is a functional ortholog of human TopBP1, and the Mec1/ATR activation mechanism is conserved from yeast to humans.

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Introduction

Eukaryotic cells have elaborate mechanisms to ensure the faithful maintenance and replication of the genome. Genotoxic stress activates a signal transduction pathway called the DNA damage response (DDR) that coordinates cell cycle transitions, DNA replication, transcription, apoptosis, and DNA repair (1). Activation of the DNA damage checkpoint pathway provides cells time to repair DNA damage before dividing, preventing the accumulation of mutations and preserving genomic stability. In *S. cerevisiae,* the PIKK (Phosphatidylinositide 3-kinase related kinase) kinase Mec1, the mammalian ATR ortholog, senses DNA damage and replication stress and initiates the DNA damage response (2). Mec1 phosphorylates substrates involved in DNA replication and repair, cell cycle checkpoints, RNA metabolism, and transcription (3).

Activation of the DNA damage response requires the co-localization of two checkpoint complexes to damaged chromatin. Mec1 is localized to DNA damage through its associated partner, Ddc2, which binds to RPA-coated ssDNA (4-7). Independently of Mec1-Ddc2, the 9-1-1 checkpoint clamp complex composed of Ddc1-Mec3-Rad17 (Rad9-Hus1-Rad1 in mammalian cells) also localizes to sites of DNA damage (8-10). Forced co-localization of the Mec1-Ddc2 complex and the 9-1-1 complex to chromatin can trigger the DNA damage response in the absence of a DNA lesion (11). Furthermore, co-localization of only the Ddc1 subunit of the 9-1-1 complex with Mec1-Ddc2 is sufficient to activate to the DNA damage response (11). This suggests that Ddc1 and/or a Ddc1 interacting protein may function as a direct activator of Mec1. In fact, Ddc1 purified from yeast has been shown to modestly stimulate the kinase activity of Mec1 *in vitro* under low salt conditions (12).

Ddc1 interacts with another checkpoint protein Dpb11 (13). Dpb11 and its sequence homologs are essential for the initiation of DNA replication in eukaryotic organisms (14, 15). Specifically, Dpb11 acts as a molecular bridge between the Sld3-Cdc45-MCM helicase complex and the Sld2-DNA polymerase ε complex (16, 17). Dpb11 also has a cell cycle checkpoint function and *dpb11* mutants exhibit sensitivity to DNA damaging agents and replication stress (14, 18). In metazoans, the Dpb11 homolog TopBP1 is a general activator of ATR. A region between the sixth and seventh BRCT (BRCA1 C-terminal) domains of TopBP1 called the ATR Activation Domain (AAD) is sufficient to activate ATR-ATRIP *in vitro* and in cells (19). However, it is not clear if Mec1 is regulated in the same manner as ATR since Dpb11 lacks sequence homology to the AAD of TopBP1 and has not been shown to interact with the Mec1-Ddc2 complex.

We recently reported that a *ddc2* mutation, termed *ddc2-top*, causes sensitivity to DNA damage and replication stress and defects in Mec1 checkpoint signaling (20). Here, we took advantage of the *ddc2-top* mutant to search for a Mec1 activator protein. We found that Ddc2 interacts genetically and physically with Dpb11. Moreover, we discovered a domain of Dpb11 that is sufficient to strongly stimulate the kinase activity of Mec1. Mec1 phosphorylates Dpb11 and this phosphorylation further enhances the ability of Dpb11 to serve as a Mec1 activator. These data demonstrate that Dpb11 is a Mec1 activator linking the Mec1-Ddc2 and 9-1-1 checkpoint complexes.

Results

Dpb11 suppresses the HU sensitivity of ddc2-top

We hypothesized that the *ddc2-top* mutation disrupted an interaction between Ddc2 and a Mec1-Ddc2 activator. If so, overexpression of that protein might be expected to suppress the phenotype of *ddc2-top* yeast. To search for this protein, we overexpressed the two likely candidates, Ddc1 and Dpb11, in *ddc2-top* yeast. Dpb11 but not Ddc1 partially suppressed the hydroxyurea (HU) sensitivity caused by the *ddc2-top* mutation. This effect was greater when Dpb11 was expressed on a high-copy (2μ) plasmid compared to a low-copy (cen) plasmid (Fig. 1A). Overexpression of Dpb11 did not restore the viability of Δ*ddc2* yeast in hydroxyurea, demonstrating that the observed suppression was Ddc2-dependent (Fig. 1B). *dpb11-1* yeast are sensitive to hydroxyurea and DNA damaging agents and are defective in S-phase checkpoint signaling (14, 18), similar to the *ddc2-top* phenotype (20). Overexpression of the Dpb11-1 mutant did not suppress the HU sensitivity of *ddc2-top* yeast indicating allele-specific suppression that is consistent with a direct protein-protein interaction (Fig. 1C).

Dpb11 associates with Mec1-Ddc2

Dpb11 contains four BRCT repeats, which function in tandem as phospho-protein interacting domains. The N-terminal pair and C-terminal pair of BRCT domains bind to CDK-phosphorylated residues of Sld3 and Sld2, respectively (16, 17). *dpb11-1* encodes a nonsense mutation at residue 583, resulting in a truncation C-terminal to the BRCT domains (21) (Fig. 2A). Given that *dpb11-1* is unable to suppress to the HU sensitivity of *ddc2-top* yeast, we suspected that the C-terminal region of Dpb11 might be responsible

for an interaction with Ddc2. To determine if there is a physical interaction between Dpb11 and Ddc2, we incubated yeast lysates with recombinant Dpb11 fragments encoding the Dpb11 C-terminal domain, Dpb11-C (aa 571-764) or the other non-BRCT region of Dpb11, Dpb11-M (aa 206-325), as a control. Dpb11-C but not Dpb11-M is able to interact with Ddc2 and Mec1 (Fig. 2B). We next asked whether the *ddc2-top* mutation affected this interaction. Unlike wild-type Ddc2, Ddc2-top does not associate with Dpb11-C. Furthermore, in the presence of Ddc2-top, Mec1 is no longer able to associate with Dpb11, indicating that the observed association of Mec1 with Dpb11 is Ddc2-dependent. We conclude that Ddc2 contains a binding site for Dpb11 that is necessary for the interaction of the Mec1-Ddc2 complex with the C-terminus of Dpb11.

Dpb11 stimulates the kinase activity of Mec1-Ddc2

We next tested if Dpb11 could function as a Mec1 activator. We immunopurified Mec1-Ddc2 from yeast lysates and incubated the complexes with recombinant Dpb11 and an established substrate, MCM2 (22, 23). Addition of Dpb11 strongly stimulated the kinase activity of Mec1 in a dose-dependent manner (Fig. 3A). Dpb11-C also stimulated Mec1 kinase activity, whereas Dpb11-M did not (Fig. 3B). Thus, the Dpb11 C-terminal domain is sufficient to activate Mec1. Dpb11-C did not produce an increase in substrate phosphorylation in control immunoprecipation reactions lacking Mec1 (Fig. 3B). To confirm that the observed kinase activity was due to Mec1, we carried out the Mec1 kinase assay in the presence of Dpb11-C and increasing amounts of caffeine, an inhibitor of PIK kinases (24). Caffeine effectively inhibited the kinase activity of the activated Mec1 (Fig. 3C). These Mec1 kinase assays were performed in low salt (50 mM NaCl)

conditions. Under these conditions, Dpb11 stimulated the kinase activity of Mec1 greater than 100-fold. We wanted to determine if Dpb11 could stimulate Mec1 in the presence of a physiological salt concentration, so the Mec1 kinase assay was performed in the presence of 150 mM NaCl. In this case, the basal Mec1 kinase activity and the stimulated Mec1 kinase activity were both slightly lower, yet the degree of Dpb11-dependent activation was still very large (67-fold) (Fig. 3D). Thus, Dpb11 is a potent Mec1 activator.

Dpb11-dependent Mec1 activation requires a Ddc2-Dpb11 interaction

We previously reported that *ddc2-top* yeast have a defect in checkpoint signaling after replication stress and DNA damage (20). To determine if this defect is due to an inability of Mec1-Ddc2-top complexes to be activated by Dpb11, Mec1-Ddc2 and Mec1-Ddc2-top complexes were isolated and incubated with increasing amounts of the Dpb11-C fragment. In the presence of wild-type Ddc2, we observed increased Mec1 kinase activity with increasing amounts of Dpb11-C. However, even high concentrations of Dpb11-C did not stimulate Mec1-Ddc2-top complexes (Fig. 4). Therefore, an interaction between Ddc2 and Dpb11 is required for Dpb11 to activate Mec1. After the high-stringency washes used for the kinase assays, we did note that the Ddc2-top mutant samples contained slightly less associated Mec1 suggesting the mutation may slightly alter the affinity of Mec1 for Ddc2 (Fig. 4).

Dpb11 phosphorylation potentiates Mec1 activation

Mec1 targets its substrates at SQ/TQ motifs (3, 25). In the course of our kinase assays, we noticed that Dpb11-C itself served as Mec1 substrate. This region of Dpb11 contains a single Mec1 consensus site, threonine 731. Mutation of T731 to alanine largely abolished the phosphorylation of Dpb11 by Mec1 (Fig. 5A).

To assess the functional significance of this Dpb11 phosphorylation, we tested if T731 phosphorylation regulates the ability of Dpb11 to activate Mec1. In low-salt (50mM) conditions, Dpb11-C T731A stimulated Mec1 kinase activity approximately 3-fold less efficiently that wild-type Dpb11-C (Fig. 5A and 5B). However, at more physiologically relevant salt concentrations, Dpb11-C T731A is 100-fold less efficient at activating Mec1 compared to wild-type Dpb11-C (Fig. 5A and 5B). These data suggest that Mec1-catayzed Dpb11 T371 phosphorylation increases the ability of Dpb11 to activate the Mec1-Ddc2 complex. We also created a Dpb11-C phosphorylation-mimetic mutant at this site. The Dpb11-C T371E mutant partially restored Mec1 activation compared to the T371A mutant at the physiological salt concentration (Fig. 5A and 5B), supporting the idea that T731 phosphorylation promotes the ability of Dpb11 to activate Mec1.

Discussion

The Mec1/ATR kinase is an essential regulator of genome integrity (2). In this study, we examined how the kinase activity of Mec1 is regulated and discovered that an essential replication and checkpoint protein, Dpb11, functions as a direct Mec1 activator. Just as Dpb11 bridges the helicase and polymerase complexes during the initiation of replication, our data suggests that Dpb11 also serves as a link between the Mec1-Ddc2 kinase complex and 9-1-1 checkpoint clamp complex in the DNA damage response. Significantly, phosphorylation of Dpb11 by Mec1 increases its ability to serve as an activator providing a means of signal amplification.

The Mec1 activation domain of Dpb11 is located C-terminal to its BRCT domains. This Dpb11-C domain appears to be functionally equivalent to the TopBP1 ATR activation domain (AAD) (19) despite the lack of sequence conservation. The TopBP1 AAD binds the ATR-ATRIP complex through interactions with ATRIP and the ATR PIKK regulatory domain (PRD) (20). An *ATRIP* allele (*-top*) that eliminates TopBP1 binding to ATR-ATRIP cannot be activated. Similarly, we found an equivalent mutation in *Ddc2 (-top)* also cannot be activated by Dpb11. Given that very little sequence homology exists between Ddc2 and ATRIP, it is not surprising that the sequence of the TopBP1 AAD is not similar to the Dpb11-C domain. Though, given the similar mechanisms of ATR and Mec1 activation, we expect that Dpb11-C and the TopBP1 AAD proteins adopt a similar tertiary structure.

Both Dpb11 and TopBP1 contain a phosphorylated SQ/TQ site within their Mec1/ATR activation domains that enhance their ability to activate Mec1-Ddc2 or ATR-ATRIP complexes, respectively (19, 26). For Dpb11, our data indicates that this site

serves as a positive-feedback amplification loop for Mec1 activation. For TopBP1, it is still unclear whether ATR auto-amplifies using this mechanism; however, ATM phosphorylation of this site potentiates the ability of TopBP1 to activate ATR (27).

A recent publication demonstrated that the loading of the 9-1-1 clamp onto DNA can stimulate the phosphorylation of Mec1 substrates *in vitro* (12). Since Mec1-Ddc2 also associates with DNA (28), the loaded clamp may serve as a scaffold for the recruitment f other Mec1 substrates. Purified Ddc1 can also modestly stimulate Mec1 kinase activity, but only when the kinase reactions are performed in low salt conditions (12). Ddc1 associates with Mec1-Ddc2; however, the sites of interaction on Ddc1 and Mec1-Ddc2 have not been identified. We have found that recombinant Dpb11 greatly stimulates Mec1-Ddc2 kinase activity even in physiological salt concentrations. Furthermore, we identified mutations in Dpb11 and in Mec1-Ddc2 that disrupt their interactions. Taken together with our previous characterization of the *ddc2-top* mutant (20) and the work of others on the *dpb11-1* mutant (14, 18), these data indicate that the interaction between Dpb11 and Mec1-Ddc2 is critical for Mec1 checkpoint signaling and cellular resistance to DNA damage and replication stress. It will be interesting to examine the simultaneous effect of Dbp11 and Ddc1 in Mec1 kinase assays. If these proteins have distinct modes of interaction with Mec1-Ddc2, then there might be an additive effect on Mec1 activation. On the other hand, if they utilize similar binding surfaces on Mec1-Ddc2, then competition between these proteins might be expected.

Our data are is consistent with the following dual sensor and dual amplification model for Mec1 activation (Fig. 5C). RPA-coated ssDNA, generated as a consequence of replication stress or DNA damage, independently recruits the Mec1-Ddc2 and 9-1-1

complexes (7-10). The co-localization of these two complexes allows Ddc1 to initially stimulate Mec1. Mec1 then phosphorylates residues on Ddc1 necessary for the recruitment of Dpb11 (13, 29), which allows Mec1 to phosphorylate Dpb11. The phosphorylated form of Dpb11 serves as a potent Mec1 activator to further amplify Mec1 kinase activity towards its substrates. Once Mec1 is fully activated, the phosphorylation of additional checkpoint proteins, such as Rad9 and Mrc1, facilitates the recruitment of additional Mec1 substrates.

In conclusion, Dpb11 is a Mec1 activator. Activation requires Ddc2 due to a direct protein-protein interaction between Dpb11 and Ddc2. Moreover, Mec1 activation results from a positive feedback loop whereby Mec1 phosphorylation of Dpb11 stimulates its ability to activate Mec1. Thus, Dpb11 is a functional orthologue of human TopBP1 and the Mec1/ATR activation mechanism is conserved from yeast to humans.

Methods

Kinase assays

Yeast expressing HA-Mec1 and myc-Ddc2 were spheroplasted using Quantazyme lyticase (Qbiogene). Spheroplasts were lysed in 50 mM HEPES (pH 7.4), 100 mM KCl, 0.1 mM EDTA, 0.2% Tween 20, 1 mM diothiothreitol, 5 ug/mL aprotinin, 5 ug/mL leupeptin, 1 mM NaF, 50 mM β-glycerolphosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride. Mec1-Ddc2 complexes were immunoprecipitated from cleared lysate using anti-myc 9E10 antibody (Covance) and protein G agarose beads (Invitrogen). Immunoprecipitates were processed and used for kinase reactions as described (20). Where indicated, kinase reactions contained 150 mM NaCl instead of 50 mM NaCl. Quantification of kinase assays was performed using a FLA-5100 phosphorimager (Fuji Film).

Protein interactions

GST-tagged Dpb11 fragments were purified from *E. coli* with glutathione Sepharose 4B beads according to the manufacturers instructions (GE Healthcare). Yeast were harvested and lysed in low salt buffer (20 mM HEPES-KOH [pH 7.5], 0.1% Tween 20, 20% glycerol, 1 mM diothiothreitol, 5 ug/mL aprotinin, 5 ug/mL leupeptin, 1 mM NaF, 50 mM β-glycerolphosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride) using glass beads. Lysates was cleared by centrifugation and an equal volume of high salt buffer (20mM HEPES-KOH [pH 7.5], 350 mM NaCl, 25% glycerol, 1mM diothiothreitol) was added. Then, lysates were incubated with the GST-tagged protein bound to glutathione beads overnight at 4°C. Beads were washed four times in wash

buffer (25 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20, 10% glycerol, 1 mM diothiothreitol, 5 ug/mL aprotinin, 5 ug/mL leupeptin, 1 mM NaF, 50 mM β-glycerolphosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride). Bound proteins were eluted in 2X SDS sample buffer, and processed for SDS-PAGE.

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Author contributions

D.M. designed and performed research. D.M. and D.C. analyzed data and wrote the paper.

The authors declare no conflict of interest.

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Figure Legends:

Figure 1. Overexpression of Dpb11 but not Ddc1 suppresses the HU sensitivity of *ddc2-top*. (A) YDM003, a Δ*ddc2* Δ*sml1* yeast strain carrying pDM158 (a *URA3 CEN* plasmid expressing *ddc2-top* under its endogenous promoter) was transformed with galactose-inducible Dpb11 or Ddc1 high-copy (2μ) or low-copy (cen) expression vectors or an empty vector control. Cells were grown to mid-log phase and serial dilutions were spotted onto galactose plates with the indicated concentration of hydroxyurea (HU) and incubated at 30°C. (B and C) The HU sensitivity of Δ*ddc2* Δ*sml1* or *ddc2-top* yeast strains containing galactose-inducible Dpb11, Dpb11-1, or an empty *TRP1* plasmid as indicated were compared on galactose plates as in (A).

Figure 2. The C-terminus of Dpb11 interacts with Mec1-Ddc2. (A) Schematic of wild-type Dpb11, the Dpb11-1, Dpb11-M, and Dpb11-C proteins used in this study. Boxes indicate BRCT domains. (B) Yeast protein extracts from cells expressing Ddc2 or Ddc2-top were incubated with recombinant GST-tagged Dpb11 fragments bound to glutathione beads. Proteins bound to the beads were eluted, separated by SDS-PAGE, and immunoblotted with antibodies to detect Mec1, Ddc2, and the GST-tagged Dpb11 fragments.

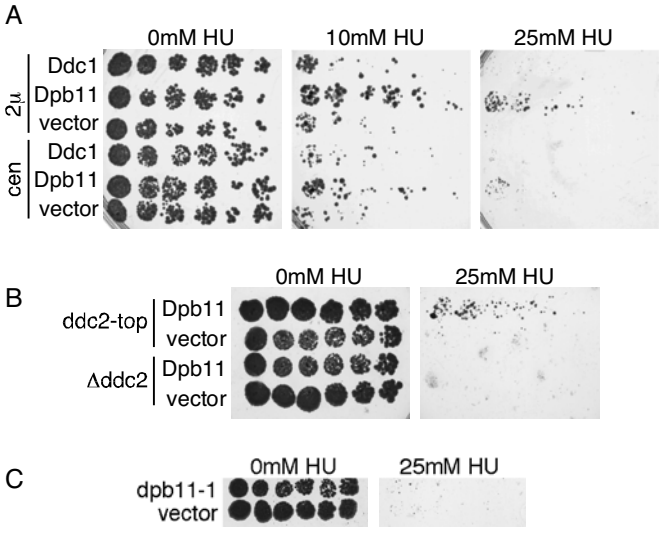
Figure 3. Dpb11 activates Mec1-Ddc2. (A) HA-Mec1-myc-Ddc2 complexes were immunopurified from yeast lysates and were incubated with recombinant Dpb11, substrate, and γ-^{32}P ATP. Kinase reactions were separated by SDS-PAGE, stained with coomassie blue (CB) to visualize the amount of substrate, and exposed to film (autorad).

A duplicate gel was immunoblotted with anti-HA and anti-myc antibodies to detect Mec1 and Ddc2, respectively (WB). (B) Mec1-Ddc2 complex kinase reactions were performed in the absence (-) or presence of recombinant Dpb11-M or Dpb11-C fragments. Anti-IgG antibody was used a control for the immunopurifications. (C) Mec1-Ddc2 complex kinase reactions containing Dpb11-C were performed in the presence of 0, 0.2, 1, and 5 mM caffeine. (D) Mec1-Ddc2 complex kinase reactions were performed with buffer containing 50mM or 150 mM NaCl. Substrate phosphorylation was quantitated using a phosphorimager.

Figure 4: Ddc2-top does not support Mec1 activation by Dpb11. Mec1-Ddc2 or Mec1-Ddc2-top complexes were purified and incubated with increasing amounts of Dpb11-C, substrate, and γ-^{32}P ATP. Kinase reactions were separated by SDS-PAGE, stained with comassie blue (CB) to visualize the substrate, and exposed to film (autorad). Both long and short exposures of the autoradiogram are shown. A duplicate gel was blotted and probed with anti-HA and anti-myc antibodies to detect Mec1 and Ddc2, respectively (WB).

Figure 5: Dpb11 Thr-731 phosphorylation potentiates Mec1 activation. (A) Mec1-Ddc2 complexes were purified and incubated with wild-type or mutant forms of Dpb11-C, substrate, and γ-^{32}P ATP. Kinase reactions were separated by SDS-PAGE, stained with coomassie blue (CB) to visualize the substrate, and exposed to film (autorad). A duplicate gel was blotted and probed with anti-HA and anti-myc antibodies to detect Mec1 and Ddc2, respectively. An anti-GST antibody was used to detect Dpb11-C

proteins (WB). (B) Quantification of substrate phosphorylation corresponding to the numbered lanes in (A). (C) Simplified model of Mec1-Ddc2 activation described in the text.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0mM HU
10mM HU
25mM HU
2μ
Ddc1
Dpb11
vector
cen
Ddc1
Dpb11
vector
B
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ddc2-top
Dpb11
vector
Δddc2
Dpb11
vector
C
0mM HU
25mM HU
dpb11-1
vector

Figure 1

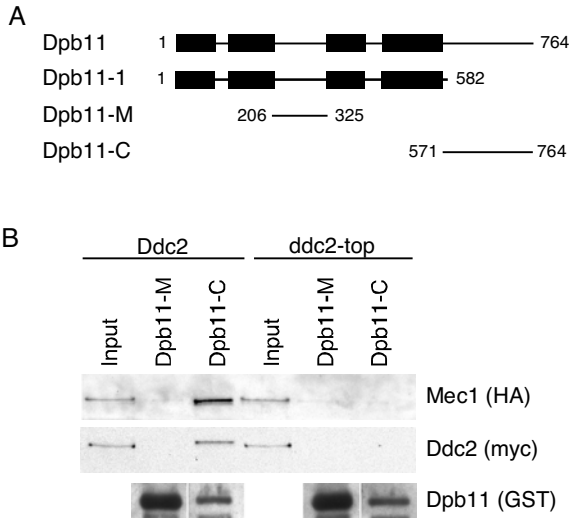
A
Dpb11
1
764
Dpb11-1
1
582
Dpb11-M
206
325
Dpb11-C
571
764
B
Ddc2
ddc2-top
Input
Dpb11-M
Dpb11-C
Input
Dpb11-M
Dpb11-C
Mec1 (HA)
Ddc2 (myc)
Dpb11 (GST)

Figure 2

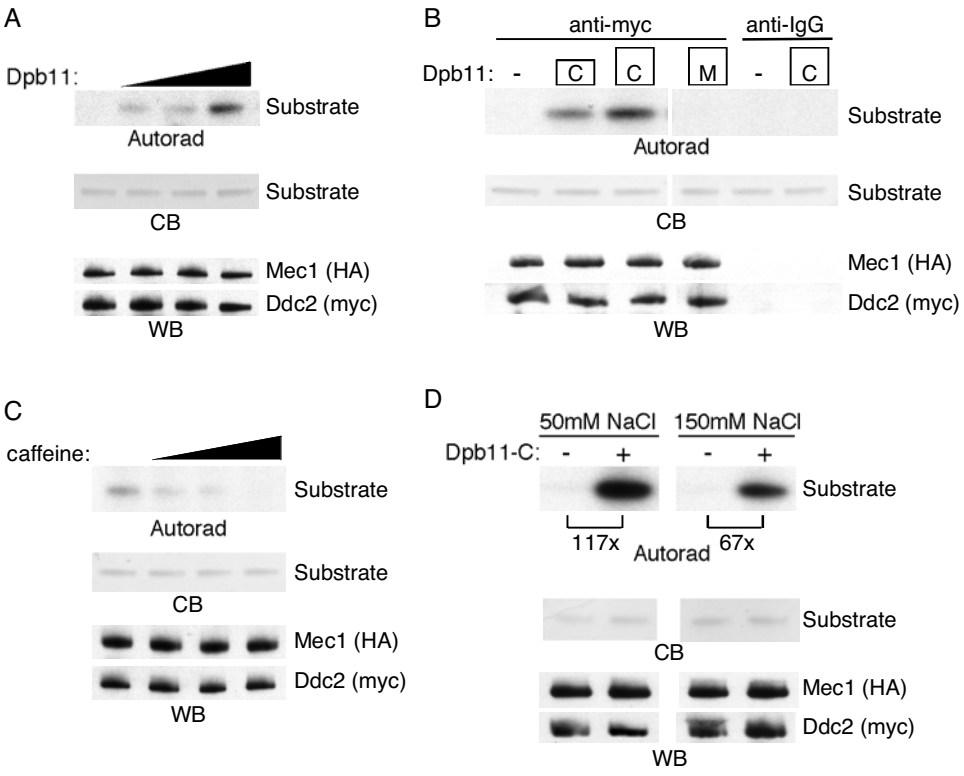
A
Dpb11:
Substrate
Autorad
Substrate
CB
Mec1 (HA)
Ddc2 (myc)
WB
B
anti-myc
anti-IgG
Dpb11: - C C M - C
Substrate
Autorad
Substrate
CB
Mec1 (HA)
Ddc2 (myc)
WB
C
caffeine:
Substrate
Autorad
Substrate
CB
Mec1 (HA)
Ddc2 (myc)
WB
D
50mM NaCl
150mM NaCl
Dpb11-C: - + - +
Substrate
117x
Autorad
67x
Substrate
CB
Mec1 (HA)
Ddc2 (myc)
WB

Figure 3

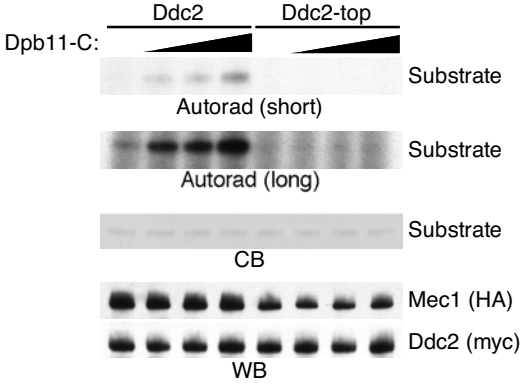
Ddc2
Ddc2-top
Dpb11-C:
Substrate
Autorad (short)
Substrate
Autorad (long)
Substrate
CB
Mec1 (HA)
Ddc2 (myc)
WB

Figure 4

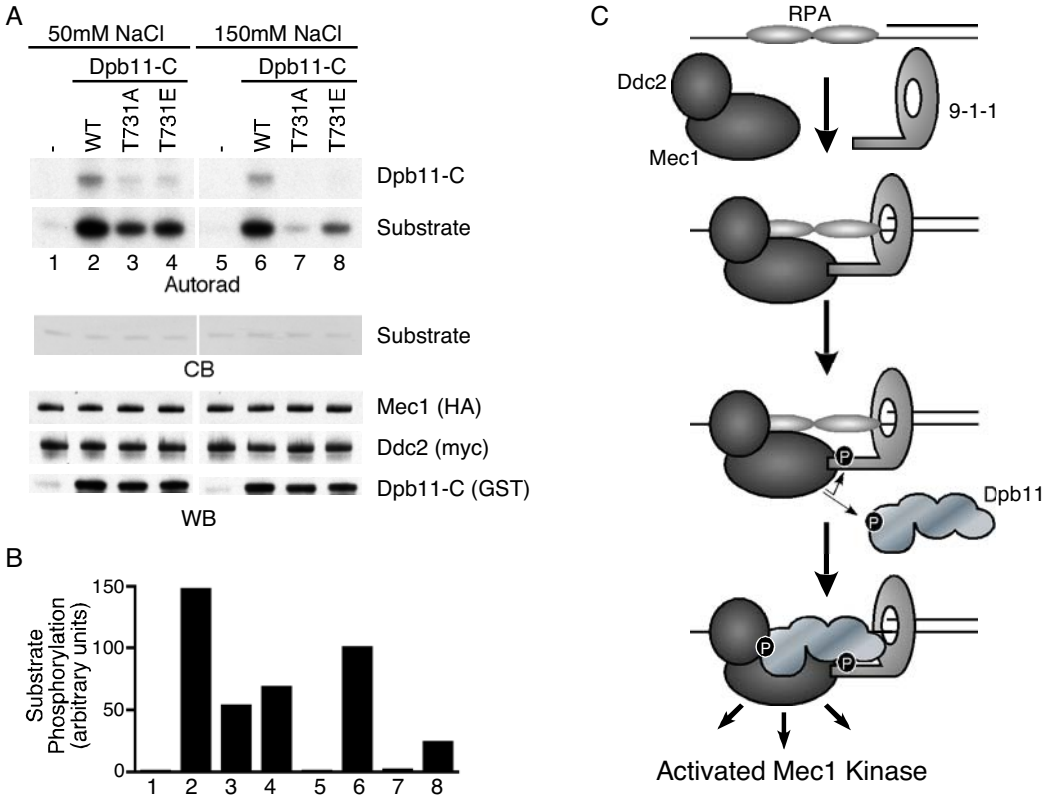
A
50mM NaCl
150mM NaCl
Dpb11-C
Dpb11-C
-
WT
T731A
T731E
-
WT
T731A
T731E
Dpb11-C
Substrate
1 2 3 4 5 6 7 8
Autorad
Substrate
CB
Mec1 (HA)
Ddc2 (myc)
Dpb11-C (GST)
WB
B
Substrate Phosphorylation (arbitrary units)
150
100
50
0
1 2 3 4 5 6 7 8
C
RPA
Ddc2
9-1-1
Mec1
P
Dpb11
P
P
P
Activated Mec1 Kinase

Figure 5

Table 1. Yeast strains used in this study.

Strain	Description	Reference or source
DMP2995/1B	*MAT*a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sml1Δ::KanMX4 ddc2Δ::KanMX4*	(4)
yDM003	DMP2995/1B [pDM158:*myc-ddc2-top-URA3-CEN*]	this study
yDM011	yDM003 [pDM172:*pGAL-Dpb11-TRP1-CEN*]	this study
yDM012	yDM003 [pDM173:*pGAL-Ddc1-TRP1-CEN*]	this study
yDM013	yDM003 [pDM174:*pGAL-Dpb11-TRP1-2μ*]	this study
yDM014	yDM003 [pDM175:*pGAL-Ddc1-TRP1-2μ*]	this study
yDM016	yDM003 [p1216:*TRP1-CEN*]	this study
yDM017	yDM003 [p1221:*TRP1-2μ*]	this study
yDM020	DMP2995/1B [pDM174:*pGAL-Dpb11-TRP1-2μ*] [p1220:*URA3-CEN*]	this study
yDM022	DMP2995/1B [p1221:*TRP1-2μ*] [p1220:*URA3-CEN*]	this study
yDM029	yDM003 [pDM183:*pGAL-dpb11-1-TRP1*-2μ]	this study
HSY1597	*MAT*a *ade2-1 ura3-1 his3-11*,*15 trp1-1 leu2-3*,*112 can1-100 MEC1-HA::sml1Δ HIS3 ddc2Δ::HGR*	(30)
yDM007	HSY1597 [pDM158:*myc-ddc2-top-URA3-CEN]*	this study
yDM010	HSY1597 [pNML1:*myc-DDC2-URA3-CEN*]	this study